

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
28 August 2003 (28.08.2003)

PCT

(10) International Publication Number
WO 03/070083 A2(51) International Patent Classification: **A61B**Clinton, MA 01510 (US). **WEST, Michael, D.** [US/US];
107 Middle Road, Southborough, MA 01772 (US).

(21) International Application Number: PCT/US03/04244

(74) Agents: **STEFFEY, Charles, E.** et al.; P.O. Box 2938,
Minneapolis, MN 55402 (US).

(22) International Filing Date: 13 February 2003 (13.02.2003)

(25) Filing Language: English

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VC, VN, YU, ZA, ZM, ZW.

(26) Publication Language: English

(30) Priority Data:
60/357,328 15 February 2002 (15.02.2002) US
PCT/US02/25175 8 August 2002 (08.08.2002) US(71) Applicant (*for all designated States except US*): **COR-
NELL RESEARCH FOUNDATION, INC.** [US/US]; 20
Thornwood Drive, Suite 105, Ithaca, NY 14850 (US).(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicants and

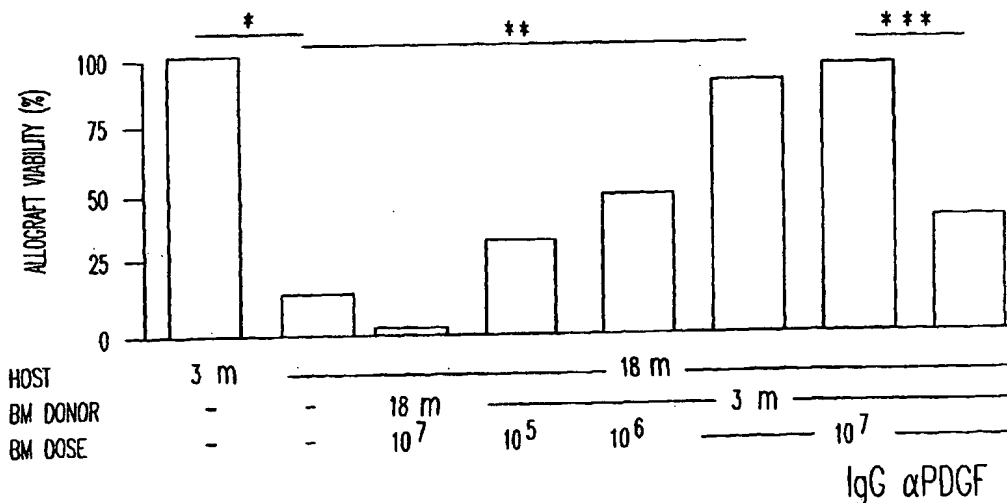
(72) Inventors: **EDELBERG, Jay, M.** [US/US]; 430 East
63rd Street, Apartment 8A, New York, NY 10021 (US).
RAFII, Shahin [US/US]; 24 Clover Drive, Great Neck,
NY 11021 (US). **HONG, Mun, K.** [US/US]; 171 East
84th Street, Apartment 28C, New York, NY 10028 (US).
LANZA, Robert, P. [US/US]; 35 South Meadow Road,

Published:

— *without international search report and to be republished
upon receipt of that report*

[Continued on next page]

(54) Title: ENDOTHELIAL PRECURSOR CELLS FOR ENHANCING AND RESTORING VASCULAR FUNCTION



(57) Abstract: The invention provides methods of treating and preventing loss of tissue vascularization that can occur, for example, upon aging.

BEST AVAILABLE COPY

WO 03/070083 A2



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**ENDOTHELIAL PRECURSOR CELLS
FOR ENHANCING AND RESTORING VASCULAR FUNCTION**

Cross-Reference to Related Applications

10

This Application claims priority to International Application No. PCT/US02/25175 filed August 8, 2002 entitled "PLATELET DERIVED GROWTH FACTOR PROTECTION OF CARDIAC MYOCARDIUM" and U.S. Provisional Patent Application Serial No. 60/357,328 filed February 15, 2002, entitled "ENHANCED DERIVATION OF CARDIAC MYOCYTES FROM BONE MARROW".

15

Government Funding

The invention described herein was made with United States Government support under Grant Numbers HL59312, AG20918 and HL67839 awarded by the National Institutes of Health. The United States Government has certain rights in this invention.

20

Field of the Invention

The invention relates to bone marrow cells, cloned endothelial precursor cells and the stem cells from which they are derived. Such cells can be genetically modified to express useful gene products. The invention further relates to methods for using these cells for treating vascular diseases, including heart disease and atherosclerosis.

25

30

Background of the Invention

In the United States and Western Europe, cardiovascular disease and its associated maladies, dysfunctions and complications are a principal cause of

disability and the chief cause of death. One specific entity significantly contributing to this pathophysiologic process is atherosclerosis, which has been generally recognized as the leading health care problem both with respect to mortality and health care costs. The American Heart Association estimates that 5 953,110 persons died of cardiovascular diseases in 1997 (41.2 percent of all deaths), more than the number of mortality for cancer (539,377), accidents (95,644) and HIV (16,516) combined. Furthermore, the American Heart Association calculates that close to a quarter of the population of the United States suffers from one or more forms of cardiovascular disease. American Heart 10 Assoc., 2000, www.americanheart.org/Heart_and_Stroke_A_Z_Guide/cvds.html. Moreover, the medical costs associated with coronary heart disease are estimated at \$95 billion dollars a year. Gonzalez & Kannewurf, 55 (19) American Journal of Health-System Pharmacy S4-7 (Supp. 1, 1998).

Atherosclerosis is a disease characterized by the deposition of fatty 15 substances, primarily cholesterol, and subsequent fibrosis in the inner layer (intima) of an artery, resulting in plaque deposition on the inner surface of the arterial wall and degenerative changes within it. The ubiquitous arterial fatty plaque is the earliest lesion of atherosclerosis and is a grossly flat, lipid-rich atheroma consisting of macrophages (white blood cells) and smooth muscle 20 fibers. The fibrous plaque of the various forms of advanced atherosclerosis has increased intimal smooth muscle cells surrounded by a connective tissue matrix and variable amounts of intracellular and extracellular lipid. At the luminal surface of the artery, a dense fibrous cap of smooth muscle or connective tissue usually covers this plaque or lesion. Beneath the fibrous cap, the lesions are 25 highly cellular consisting of macrophages, other leukocytes and smooth muscle cells. Deep in this cell-rich region may be areas of cholesterol crystals, necrotic debris and calcification.

If allowed to progress, the disease can cause narrowing and obstruction of the lumen of the artery, diminished or occluded blood flow and, consequently, 30 ischemia or infarction of the predominantly affected organ or anatomical part,

such as the brain, heart, intestine or extremities. The result can be significant loss of function, loss of cellular substance, emergency medical and/or surgical procedures, and significant disability or death. Alternatively, the arterial wall can be severely weakened by the infiltration of the muscular layer with the lipid
 5 (cholesterol), inflammatory white blood cells, connective tissue and calcium, resulting in soft and/or brittle areas which can become segmentally dilated (aneurysmal) and rupture or crack leading to organ, limb or even life-threatening hemorrhage.

Ischemic heart disease is the most common cause of morbidity and
 10 mortality in the population over the age of sixty-five. Sullivan, L.W. 1990. Healthy people 2000. *N Engl J Med.* 323:1065-1067; Wei, J.Y. 1992. Age and the cardiovascular system. *N Engl J Med.* 327:1735-1739; Association, A.H. 1993-1995. Heart and stroke facts statistical supplement/1994-1996. Dallas, TX: The Association. Elucidation of the cellular and molecular pathways that are
 15 impaired with aging is critical to the development of specific strategies to prevent and reduce the pathology of cardiovascular disease associated with advancing age.

In younger individuals, myocardial ischemia induces the development of a collateral vasculature supply that partially protects the cardiac tissue from
 20 subsequent coronary events. Hirai et al. (1989) *Circulation.* 79:791-796; Ejiri et al. (1990) *J Cardiol.* 20:31-37; Kodama et al. (1996). *J Am Coll Cardiol.* 27:1133-1139; Banerjee et al., (1993) *Int J Cardiol.* 38:263-271. However, angiogenesis is impaired in older heart and peripheral vascular beds. Hudlicka et al. (1996) *J Vasc Res.* 33:266-287; Isoyama (1994) *Drugs Aging.* 5:102-115;
 25 Tomanek et al. (1990) *Am J Physiol.* 259:H1681-1687; Anversa et al. (1994) *Am J Physiol.* 267:H1062-1073; Azhar et al. (1999) *Exp Gerontol.* 34:699-714; Rakusan et al. (1994) *Cardiovasc Res.* 28:969-972; Rivard et al. (1999) *Circulation.* 99:111-120; Reed et al. (2000) *J Cell Biochem.* 77:116-126. The etiology of the impaired angiogenic activity in the senescent heart and within
 30 aging blood vessels is not known. In fact, despite recent advances in our

understanding of the molecular pathways regulating angiogenesis during embryonic development, the mechanistic alterations in angiogenic function in the senescent vasculature are not well understood.

The etiology of atherosclerotic plaques is similarly a matter of debate and uncertainty. Much research in recent years has focused upon the molecular pathways of cholesterol deposition and upon altering serum lipoprotein concentrations for achieving therapeutic effect. Goldstein et al., Science 292: 1310-12. There has been speculation that the replicative senescence of vascular endothelium plays a role in the etiology of atherosclerosis. Chang et al., 1995 Proc. Natl. Acad. Sci. 92:11190-94. Moreover, studies show that telomere length can determine the lifespan of cells. Bodnar et al., 1998 Science 279: 349-53. In addition, cells with a morphology similar to senescent cells co-localize with mature atherosclerotic plaques. Osamu et al., 1989 Am. J. Pathol. 135: 967-76. However, methods for grafting young vascular endothelial cells into an old animal have been unavailable.

Therefore, new approaches are needed for counteracting the age-associated changes in angiogenic pathways and the repair of endothelium within senescent vascular tissues.

20 **Summary of the Invention**

According to the invention, administration of endothelial precursor cells can reverse the effects of aging on mammalian vascular tissues. Such administration can therefore be used for treating vascular diseases or conditions. Such cells have the ability to find their way to and then integrate into various tissues, including vascular tissues, bone marrow and cardiac tissues. After becoming associated with these tissues, the endothelial precursor cells can restore angiogenesis and/or generate myocytes. Moreover, such endothelial precursor cells can deliver PDGF to cardiac tissues, wherein the PDGF is also useful for restoring angiogenesis in the peripheral vasculature and in senescent cardiac tissues.

The invention therefore provides a method for treating a vascular condition in a mammal that involves administering to the mammal a therapeutically effective amount of endothelial precursor cells. In some embodiments, the endothelial precursor cells can express c-kit. In other
5 embodiments, the endothelial precursor cells can express or bind platelet derived growth factor. In other embodiments, the endothelial precursor cells can induce expression of platelet derived growth factor in neighboring (e.g. co-cultured) cells.

The vascular condition can be, for example, a myocardial infarction; in
10 this case administration of the endothelial precursor cells reduces the size of the myocardial infarction. In other embodiments, the vascular condition is atherosclerosis. The vascular condition can also be ischemia, tachycardia, congestive heart failure, peripheral vasculature disorder, hypertension, stroke, thrombosis, arrhythmia or tachycardia. While any mammal may be treated by
15 the methods of the invention, in many embodiments, the mammal is a human.

The endothelial precursor cells can be administered intravascularly, intravenously, intraarterially, intraperitoneally, via intraventricular infusion, via infusion catheter, via balloon catheter, via bolus injection, or via direct application to tissue surfaces during surgery. A therapeutically
20 effective amount of endothelial precursor cells can be, for example, about 10^2 to about 10^{10} endothelial precursor cells, or about 10^4 to about 10^9 endothelial precursor cells.

In some embodiments, the endothelial precursor cells are exposed to platelet derived growth factor AB prior to administration to the
25 mammal. According to the invention, older bone marrow normally cannot be used for treating vascular conditions, but when cultured in the presence of platelet derived growth factor, the older bone marrow takes on many of the characteristics of young bone marrow and becomes useful for treating vascular conditions.

The endothelial precursor cells can be syngeneic endothelial precursor cells originally obtained from the mammal to be treated. Administration of such cells will minimize immunological reactions that may be directed against the endothelial precursor cells. To facilitate
5 isolation of the endothelial precursor cells, the mammal can be pre-treated with G-CSF, GM-CSF, VEGF, SCF, bFGF, SDF-1, interleukin 1 or interleukin 8 before isolation of the endothelial precursor cells.

The endothelial precursor cells employed in the methods of the invention can be derived from bone marrow, peripheral blood, umbilical cord blood, liver
10 tissue or fat. The endothelial precursor cells can also be derived from an embryonic stem cell line. In other embodiments, the endothelial precursor cells are derived from at least one nuclear transfer unit formed *in vitro* by fusion of an enucleated oocyte with a somatic cell from the mammal. The endothelial precursor cells can also be derived from an inner cell mass of a blastocyst
15 generated *in vitro*.

In some embodiments, the endothelial precursor cells can comprise a heterologous DNA encoding a therapeutic agent that can be expressed in the endothelial precursor cells. Such a therapeutic agent can, for example, be a platelet derived growth factor polypeptide having any one of SEQ ID NO:1,
20 SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6. The therapeutic agent can also be a platelet derived growth factor receptor, for example, having SEQ ID NO:35 or SEQ ID NO:36. Other examples of therapeutic agents include a cytokine, a growth factor, a hormone, streptokinase, tissue plasminogen activator, plasmin, urokinase, an anti-thrombotic agent, an
25 anti-inflammatory agent, a metalloproteinase inhibitor or a nematode-extracted anticoagulant protein.

The invention further provides methods for treating or preventing a myocardial infarction in a patient having or at risk for developing a myocardial infarction. The method involves administering to the patient a

therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

The invention also provides a method for reducing the size of a myocardial infarction in a patient at risk for developing a myocardial infarction, such a method can include administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway. The size of the myocardial infarction can be measured by the extent of myocardial necrosis.

The invention also provides a method of restoring cardiac angiogenic function in a patient having senescent cardiac angiogenic function. Such a method can include administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

The invention also provides a method of restoring vascular function in a patient having peripheral vasculature disorder (PVD), wherein the method comprises administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

The invention further provides a method of restoring vascular function in or near the brain of a patient in need of such restoration, wherein the method comprises administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway. The patient may be suffering or may have suffered a stroke.

The invention further provides a method of restoring cardioplastic potential of bone marrow cells obtained from a patient having senescent cardiac angiogenic function, wherein the method comprises administering an effective amount of PDGF AB to a culture of said bone marrow cells.

The invention also provides a method of treating cardiovascular dysfunction, wherein the method comprises administering to a patient

suffering from said dysfunction, a therapeutically effective amount of cardiac myocytes, wherein said cardiac myocytes are derived from autologous stem cells and wherein said stem cells have been cultured in the presence of PDGF AB. The cardiovascular dysfunction can be at least
5 one of myocardial infarction, ischemia, peripheral vasculature disorder (PVD), stroke, arrhythmia, tachycardia, or heart failure.

The invention further provides a method of restoring cardiac angiogenic function in a patient having senescent cardiac angiogenic function, wherein the method comprises administering to the patient a
10 therapeutically effective amount of cardiac myocytes, wherein said cardiac myocytes are derived from autologous stem cells and wherein said stem cells have been cultured in the presence of PDGF AB.

The invention also provides a method of restoring cardioplastic potential of stem cells obtained from a patient having senescent cardiac
15 angiogenic function, wherein the method comprises administering to a culture of said stem cells, an effective amount of PDGF AB.

The invention further provides a method of increasing the kinetics of cardiac myocyte derivation from bone marrow cells obtained from a patient having senescent cardiac angiogenic function, wherein the method
20 involves administering to a culture of said bone marrow cells an effective amount of PDGF AB.

The invention also provides a method of increasing the kinetics of cardiac myocyte derivation from stem cells obtained from a patient having senescent cardiac angiogenic function, wherein the method comprises
25 administering to a culture of said stem cells an effective amount of PDGF AB.

The agent that restores a PDGF B dependent communication pathway can be at least one of PDGF AB, PDGF BB, PDGF A, PDGF B, stem cells, young bone marrow endothelial precursor cells, epidermal
30 growth factor or small molecule. The route of administration is by

intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stem cell, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration. The stem cells or young bone marrow, or endothelial precursor cells can be genetically modified to express a heterologous protein, RNA, or hormone. The stem cells or young bone marrow endothelial precursor cells can be genetically modified to over-express a native protein, RNA or hormone. The stem cells or young bone marrow endothelial precursor cells may also be modified to express, for example, cytokines, growth factors, hormones, signaling intermediates, sugar moieties, small molecules, anti-sense RNA, and to perform various biological actions that facilitate vascularization of senescent tissues.

The invention further provides cardiac myocytes exhibiting cardioplastic potential and derived from endothelial precursor cells obtained from a patient having senescent cardiac angiogenic function, said cardiac myocytes obtained through a process of culturing said endothelial precursor cell in the presence of an effective amount of PDGF, for example, PDGF AB or PDGF BB. Such endothelial precursor cells can be derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

The invention also provides a method of delivering platelet derived growth factor to cardiac tissues of a mammal comprising administering live endothelial precursor cells to a mammal and thereby delivering platelet derived growth factor to cardiac tissues. The platelet-derived growth factor can be PDGF B, PDGF A, PDGF AB, PDGF BB or any other form of PDGF that has activity or can combine with a PDGF polypeptide to generate an active PGDF protein.

The invention further provides a method of delivering platelet derived growth factor to cardiac tissues of a mammal comprising

administering live young bone marrow cells to a mammal and thereby delivering platelet derived growth factor to cardiac tissues.

5 The invention also provides a method of preventing myocardial necrosis comprising administering live endothelial precursor cells to a mammal and thereby delivering platelet derived growth factor B to cardiac tissues in danger of myocardial necrosis.

10 The endothelial precursor cells and/or young bone marrow cells can express platelet-derived growth factor B upon association with cardiac myocytes within the cardiac tissues. Cardiac microvascular endothelial cells within the cardiac tissues can also express platelet-derived growth factor B after administration of the endothelial precursor cells and/or the young bone marrow cells. Such endothelial precursor cells and young bone marrow cells provide sustained delivery of platelet-derived growth factor B.

15 Administration of these cells can be intravascular, intravenous, intraarterial, intraperitoneal, via intraventricular infusion, via infusion catheter, via balloon catheter, via bolus injection, or via direct application to cardiac tissue during surgery. Administration can also be local or intravenous.

20 The endothelial precursor cells are derived from autologous bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat. Such endothelial precursor cells or young bone marrow cells can also be cultured in the presence of platelet derived growth factor AB prior to administration. Endothelial precursor cells are also derived from
25 allogeneic and xenogenic bone marrow, peripheral blood, umbilical cord blood, organs, tissues or fat. They are also derived from primitive precursor stem cells including but not limited to allogeneic biparental and parthenogenetic embryonic stem cells obtained by nuclear transfer and related technologies for reprogramming somatic cells to and embryonic
30 state.

The cardiac tissues treated can be within a senescent heart. The mammal treated may have suffered from cardiovascular disease such as atherosclerosis, myocardial infarction, ischemia, tachycardia, or congestive heart failure.

5

Description of the Figures

Figure 1A provides a photograph of a gel illustrating an RT-PCR analysis of PDGF-A, PDGF-B, and β -actin expression in ventricular myocardial samples isolated from young adult (3 month) and senescent mice (18 month).

10

Figure 1B provides a photograph of a gel illustrating the expression profile of CMECs from 3- and 18-month-old mice co-cultured in transwells with fetal cardiac myocytes for zero to 3 days.

Figure 1C provides a graph illustrating the fold-change in protein levels of CMECs from 3-month-old and 18-month-old mice cultured in the presence vs. the absence of fetal cardiac myocytes for 3 days.

15

Figure 2A provides representative examples of neonatal cardiac transplants into young adult (3 months old) (n=20) and senescent hosts (18 months old) (n=17). Senescent hosts were also transplanted with silicon (n=8), neonatal lungs (n=8), and neonatal hearts after pinnal pretreatment by injection of 100 ng of VEGF (n=12) or 100 ng of PDGF-AB (n=13). An arrow indicates viable/intact transplants. The majority of the cardiac allografts transplanted into the control and VEGF pretreated senescent mice resulted in a necrotic loss of both allograft and host pinnal tissue beyond the transplant site (arrowhead). Allograft viability was scored by pinnal and transplant integrity. Cardiac allograft viability in young adult and PDGF-AB-pretreated senescent hosts was confirmed by pinnal electrocardiograms (5-s tracing). * $P < 0.01$ versus young adult; ** $P < 0.01$ versus senescent adult and $P < 0.01$ versus senescent adult treated with VEGF.

20

25

Figure 2B provides a bar graph illustrating pinnae blood in ml/100g tissue/min in untreated tissues and in tissues treated with PDGF AB. These

30

results were obtained by laser Doppler measurements of capillary blood flow in the posterior auricular circulation. Pretreatment with PDGF AB significantly increased blood flow in both the young (3 month) as well as the older (18 month) hosts.

5 Figure 2C provides a bar graph illustrating vascular density per square mm in untreated tissues and in tissues treated with PDGF AB. These results were obtained by histological measurements of vascular density in the posterior auricular circulation. Histological assessment confirmed that PDGF AB increased collateral vascular density in the pinnae of both the young and
10 senescent mice.

 Figure 3A illustrates the temporal gene expression profiles of bone marrow-derived endothelial precursor cells (EPCs) co-cultured for 0 to 48 hours with cardiac myocytes. By way of comparison, the temporal gene expression profiles of cardiac microvascular endothelial cells (CMECs) co-cultured for 0
15 and 48 hours with cardiac myocytes are shown. The top panels show the expression profiles of EPCs and CMECs isolated from 3-month-old mice, whereas the bottom panels show the expression profiles of EPCs and CMECs isolated from 18-month-old mice. As illustrated, the young EPCs and CMECs express PDGF B, whereas the older EPCs and CMECs do not.

20 Figure 3B provides a graph illustrating the PDGF-B/PECAM protein ratio in 3-month-old bone marrow-derived EPCs that were co-cultured with cardiac myocytes.

 Figure 3C provides representative photomicrographs of X-gal stained tissue sections from 18-month-old mice receiving 10^7 bone marrow cells from 3-
25 month-old *Rosa-26* (β -galactosidase (+)) mice one week before cardiac engraftment. Aging wild-type host bone marrow with young transgenic cells (a), young transgenic cell incorporation in (b through e) and around (f and g) the wild-type cardiac myocardium transplanted into the aging hosts. Co-staining for von Willebrand factor of intraallograft with arrows highlighting transgenic cells

(c through e) and periallograft pinnal tissue (g) and for PDGF-B of intraallograft tissue (h); bar = 25 μ m (a, b, f, and g) and 10 μ m (c, d, e, and h).

Figure 3 D provides representative examples of pinnal cardiac allografts in 18-month-old hosts with either no bone marrow transplantation (control) or 10^7 bone marrow cells from 3-month-old donor (BMT) one week before cardiac engraftment. The arrow indicates the location of the viable cardiac allograft. The arrowhead provides the location of necrotic loss for both cardiac allograft and host pinnal tissue.

Figure 3E provides a bar graph illustrating the viability of cardiac allografts in young and senescent control hosts (3-month-old, 8/8; 18-month-old, 1/8) and senescent hosts receiving bone marrow cells isolated from 18-month-old donors (10^7 cells, 0/6), and from 3-month-old donors (10^5 cells, 2/6; 10^6 cells, 6/12; and 10^7 cells, 15/16) alone or with pinnal antibody pretreatment (IgG, 7/7; anti-PDGF-AB, 3/7). * P <0.05 3-month-old vs. 18-month-old transplant hosts; ** P <0.05 18-month-old hosts control vs. transplantation with 3 month-old bone marrow; *** P <0.05 IgG vs. anti-PDGF AB.

Figure 4A provides representative photographs of Masson's trichrome stained sections of 4-month-old rat hearts pretreated with PBS or PDGF-AB for 24 h before LAD ligation.

Figure 4B provides a graph showing the myocardial infarct size scored 14 days after coronary artery ligation (control, n=13; PDGF-AB, n=12). * P <0.02, PDGF vs. control.

Figure 4C provides representative photographs of Masson's trichrome staining in 24-month-old rat hearts pretreated with PBS or PDGF-AB 24 h before LAD ligation.

Figure 4D provides a graph showing myocardial infarct size 14 days after coronary ligation (control, n=5; PDGF-AB, n=7). * P <0.03, PDGF vs. control.

Figure 5A provides a photomicrograph of a representative 3-month-old bone marrow-derived cardiac myocyte aggregate that exhibited spontaneous chronotropy after 4 weeks of tissue culture: parallel bars represent single cell

diastole (outside bars) and systole (inside bars) ($15 \pm 4\%$ cell volume change) (bar= $10\mu\text{m}$) (movie on disc).

Figure 5B provides a photomicrograph of representative 3-month-old bone marrow-derived cell cultures immunostained for troponin T (bar= $25\mu\text{m}$).

5 Figure 6A provides a representative gel of RT-PCR products illustrating temporal gene expression of 3-month-old bone marrow-derived cells.

Figure 6B provides a representative gel of RT-PCR products illustrating temporal gene expression of 3-month-old bone marrow-derived cells in the presence and absence of exogenous PDGF.

10 Figure 6C provides a representative graph of *in vivo* chronotropic activity as a function of time in a 3-month-old bone marrow-derived cardiac myocyte before and after adrenergic stimulation.

Figure 7A provides a representative gel of RT-PCR products illustrating temporal gene expression profiles of 18-month-old bone marrow-derived cells.

15 Figure 7B provides a representative gel of RT-PCR products illustrating αMHC expression in 18-month-old bone marrow-derived cells in the presence and absence (control) of exogenous PDGF. As a further control, $\beta\text{-actin}$ expression was also observed

Figure 7C provides a representative graph of *in vivo* chronotropic activity as a function of time in a PDGF-induced 18-month-old bone marrow-derived cardiac myocyte before and after adrenergic stimulation.

20

Detailed Description of the Invention

The present invention provides pharmaceutical compositions comprising an effective amount of endothelial precursor cells, for example, stem cells, embryonic endothelial cells, embryonic stem cell lines, hematopoietic stem cells, young adult bone marrow cells or older bone marrow cells that have been treated with platelet derived growth factor. The invention is also directed to methods for treating a vascular condition or a vascular disease in a mammal that include administering an effective amount of endothelial precursor cells, for example,

25

30

endothelial precursor cells that express c-kit or platelet derived growth factor B. Such cells can be administered alone or in combination with platelet-derived growth factor AB (PDGF AB). Moreover, the cells can be genetically engineered to express useful gene products that can further enhance restoration and health of aging vascular tissues.

According to the invention, young endothelial precursor cells home to sites of angiogenesis in aging mammalian vessels. The ability of the young bone marrow to augment the population of aging bone marrow is illustrated by experiments described herein where LacZ+, *Rosa-26* bone marrow was transplanted intravenously into intact isogeneic older mice 1 week before inducing cardiac angiogenesis. Analysis of these mice revealed that the genetically marked bone marrow (β -galactosidase-positive) cells were recruited to and engrafted within the senescent bone marrow. Hence, transplanted endothelial precursor cells become available to facilitate angiogenesis.

Moreover, further experiments described illustrate that while cardiac allografts transplanted into older mice did not become vascularized, transplantation of young bone marrow cells into old mice restored the vascularization and function of such exogenous cardiac tissue. Similar experiments where the bone marrow of old mice was transplanted failed to reverse the aging-associated decline in cardiac angiogenic function. The restoration of the senescent vascular function by the young bone marrow cells was dose-dependent in that greater numbers of young bone marrow cells improved the functioning of older vascular tissues.

In another embodiment, the invention provides pharmaceutical compositions of bone marrow cells that have been treated with PDGF. According to the invention, while bone marrow from senescent mammals fails to generate cardiac myocytes, exposure to PDGF AB restores the ability of such older bone marrow cells to generate myocytes. Such older bone marrow cells can thus be removed from a patient suffering, or in danger of suffering, from a vascular disease, the bone marrow cells can be cultured with platelet derived

growth factor and then these cultured cells can be introduced back into the patient to treat or prevent the vascular disease. During this process, these bone marrow cells can also be genetically engineered to express useful gene products that can further enhance restoration and health of aging vascular tissues, particularly within the heart.

Hence, the invention provides compositions comprising cells capable of improving the function of older vascular tissues and methods for treating a variety of vascular diseases. In some embodiments, the compositions and methods of the invention promote angiogenesis and/or re-endothelialization. As used herein, angiogenesis is a process in which endothelial cells form a vascular bed to provide blood to organs through the body, including the heart. Re-endothelialization refers to the homing of circulating endothelial precursor cells to sites of intimal injury such as occurs in atherosclerotic plaques.

15 **Vascular Diseases**

The vascular diseases treated by the present invention are vascular diseases of mammals. The word mammal means any mammal. Some examples of mammals include, for example, pet animals, such as dogs and cats; farm animals, such as pigs, cattle, sheep, and goats; laboratory animals, such as mice and rats; primates, such as monkeys, apes, and chimpanzees; and humans. In some embodiments, humans are preferably treated by the methods of the invention.

According to the invention, endothelial cells within normal vascular tissues change as they grow older, exhibiting reduced angiogenesis, reduced capacity for re-endothelialization and losing their ability to communicate with other cells by secreting signaling agents. These changes can lead to a diminished capacity for blood vessel formation, a reduction in blood flow to the associated organ or system, and an inability to recover from injuries or diseases that adversely affect blood vessels.

Accordingly, the invention relates to methods for treating endothelial dysfunction, or a vascular condition, or a circulatory condition, such as a condition associated with loss, injury or disruption of the vasculature within an anatomical site or system. The term "vascular condition" or "vascular disease" refers to a state of vascular tissue where blood flow is, or can become, impaired.

Many pathological conditions can lead to vascular diseases that are associated with alterations in the normal vascular condition of the affected tissues and/or systems. Examples of vascular conditions or vascular diseases to which the methods of the invention apply are those in which the vasculature of the affected tissue or system is senescent or otherwise altered in some way such that blood flow to the tissue or system is reduced or in danger of being reduced. Examples of vascular conditions that can be treated with the compositions and methods of the invention include atherosclerosis, preeclampsia, peripheral vascular disease, erectile dysfunction, cancers, renal failure, heart disease, and stroke. Vascular, circulatory or hypoxic conditions to which the methods of the invention apply also include those associated with, but not limited to, maternal hypoxia (e.g., placental hypoxia, preeclampsia), abnormal pregnancy, peripheral vascular disease (e.g., arteriosclerosis), transplant accelerated arteriosclerosis, deep vein thrombosis, erectile dysfunction, cancers, renal failure, stroke, heart disease, sleep apnea, hypoxia during sleep, female sexual dysfunction, fetal hypoxia, smoking, anemia, hypovolemia, vascular or circulatory conditions which increase risk of metastasis or tumor progression, hemorrhage, hypertension, diabetes, vasculopathologies, surgery (e.g., per-surgical hypoxia, post-operative hypoxia), Raynaud's disease, endothelial dysfunction, regional perfusion deficits (e.g., limb, gut, renal ischemia), myocardial infarction, stroke, thrombosis, frost bite, decubitus ulcers, asphyxiation, poisoning (e.g., carbon monoxide, heavy metal), altitude sickness, pulmonary hypertension, sudden infant death syndrome (SIDS), asthma, chronic obstructive pulmonary disease (COPD), congenital circulatory abnormalities (e.g., Tetralogy of Fallot) and Erythroblastosis (blue baby syndrome). In particular embodiments, the

invention is a method of treating loss of circulation or endothelial dysfunction in an individual.

Thus, the invention is directed to methods of treating diseases such as stroke, atherosclerosis, acute coronary syndromes including unstable angina, thrombosis and myocardial infarction, plaque rupture, both primary and secondary (in-stent) restenosis in coronary or peripheral arteries, transplantation-induced sclerosis, peripheral limb disease, intermittent claudication and diabetic complications (including ischemic heart disease, peripheral artery disease, congestive heart failure, retinopathy, neuropathy and nephropathy), or thrombosis.

In some embodiments, the vascular condition or vascular disease arises from damaged myocardium. As used herein "damaged myocardium" refers to myocardial cells that have been exposed to ischemic conditions. These ischemic conditions may be caused by a myocardial infarction, or other cardiovascular disease. The lack of oxygen causes the death of the cells in the surrounding area, leaving an infarct that can eventually scar.

Preferably, damaged myocardium is treated with the methods and compositions of the invention before damage occurs (e.g. when damage is suspected of occurring) or as quickly as possible after damage occurs. Hence, the methods and compositions of the invention are advantageously employed on aged heart tissues that are in danger of ischemia, heart attack or loss of blood flow. The methods and compositions of the invention are also advantageously employed on recently damaged myocardium and on not so recently damaged myocardium.

As used herein "recently damaged myocardium" refers to myocardium that has been damaged within one week of treatment being started. In a preferred embodiment, the myocardium has been damaged within three days of the start of treatment. In a further preferred embodiment, the myocardium has been damaged within twelve hours of the start of treatment.

The methods and compositions of the invention can be used to prevent or to treat these vascular conditions. These methods involve administering an effective amount of endothelial precursor cells, for example, stem cells, young bone marrow cells, hematopoietic stem cells, embryonic stem cell lines or young cardiac microvascular endothelial cells. Such cells can be administered alone or in combination with platelet-derived growth factor (PDGF). Such an effective amount is effective when it stimulates the generation of myocytes or restores some vascularization in a tissue.

10 Isolating Endothelial Precursor Cells

According to the invention, endothelial precursor cells, hematopoietic stem cells, erythropoietic stem cells and other types of stem cells can reverse age-related defects in cardiac angiogenesis. As used herein, the term "endothelial precursor cells" includes all types of cells that can promote neogenesis or angiogenesis of vascular tissues. Examples include endothelial precursor cells, endothelial cell precursors, hematopoietic stem cells, embryonic stem cell lines, erythropoietic stem cells, young bone marrow cells, young cardiac microvascular endothelial cells and other types of stem cells. Such endothelial precursor cells are capable of populating the intact, senescent bone marrow, homing to sites of cardiac angiogenic induction, restoring pathways required for vascular function, homing to sites of intimal injury and facilitating re-endothelialization. These cells can restore and stimulate cardiac angiogenesis in an aging host, for example, by healing injured vascular tissues, reducing the size of atherosclerotic lesions, stimulating angiogenesis, generating cardiac myocytes and promoting formation of new blood vessels and new endothelial tissues.

The endothelial precursor cells employed in the invention can be stem cells or partially differentiated endothelial precursor cells. The term endothelial cell precursors is used interchangeably herein with endothelial precursor cells. Because endothelial cell precursors are present in circulating blood, they are also

referred to as circulating endothelial precursor cells (see U.S. Patent Application No. 60/349,345, the priority of which is claimed, and Lyden et al., 2002, supra). Such stem cells and endothelial precursor cells can be derived from nuclear transfer-derived embryonic cells from pre-implantation embryos, from in vitro fertilized embryos, parthenogenetic embryos or aborted fetuses, from young adult bone marrow-derived cells, and/or from adult stem cells.

Pluripotent stem cells are capable of developing into more than two types of mature cells, such as endothelial cells, hematopoietic cells, and at least one other type of cells. Bipotent stem cells are capable of developing into two types of mature cells, such as endothelial cells and hematopoietic cells. Progenitor cells are capable of developing into one type of mature cells, such as endothelial cells or hematopoietic cells. Pluripotent stem cells, bipotent stem cells, and progenitor cells are capable of developing into mature cells either directly, or indirectly through one or more intermediate stem or progenitor cell. An endothelial stem cell is a stem cell that is capable of maturing into at least one type of mature endothelial cell. The endothelial stem cell may be pluripotent, bipotent, or monopotent. Monopotent endothelial stem cells are also referred to as endothelial progenitor cells

Pluripotent endothelial stem cells are capable of developing into mature endothelial cells and at least two other types of cells. Bipotent endothelial stem cells are capable of developing into mature endothelial cells and one other type of cells, such as hematopoietic cells. Monopotent endothelial cells, i.e. endothelial progenitor cells, are capable of developing into mature endothelial cells.

According to the invention, the term endothelial precursor cells always includes progenitor cells that can differentiate into endothelial precursor cells and/or endothelial cells. Hence, any population of stem cells (pluripotent, bipotent, monopotent, etc.) or precursor cell types can be used in the invention so long as they can generate endothelial cells. Thus, hematopoietic stem cells

differentiate to form endothelial cell precursors, and endothelial cell precursors give rise to endothelial cells.

Hematopoietic stem cells and endothelial cell precursors can be isolated directly from bone marrow, fetal liver, circulating peripheral blood, and autologous umbilical cord blood. The leukocyte fraction of peripheral blood is a useful source of endothelial cell precursors. In addition, endothelial cell precursors can be produced in vitro or in vivo through the differentiation of hematopoietic stem cells. For example, in addition to giving rise to cells such as B and T lymphocytes, granulocytes, and monocytes, hematopoietic stem cells isolated from adult human bone marrow also differentiate into non-hematopoietic lineages (lin⁻) that give rise to endothelial cell precursors (Otani et al., Nature Medicine, 2002, 8(9): 1004-1010).

Endothelial precursor cells can be identified by their surface antigens and/or by the factors they express. Such antigens include, for example, one or more vascular endothelial growth factor receptors (VEGFR). Examples of VEGFRs include FLK-1 and FLT-1. The FLK-1 receptor is also known by other names, such as VEGFR-2. Human FLK-1 is sometimes referred to in the literature and herein as KDR. Bone-marrow reconstituting hematopoietic stem cells and endothelial cell precursors both have the CD-34 antigenic determinant (U.S. Patent No. 5,980,887, supra.) and express vascular endothelial growth factor receptor-1 (VEGFR-1) (Lyden et al., 2001, supra.). Endothelial cell precursors and vascularizing endothelial cells both express vascular endothelial growth factor receptor-2 (VEGFR-2) (Neithammer et al., 2002, supra.).

At least some endothelial precursor cells also express the CD34⁺ marker. The endothelial precursor cells may be further characterized by the absence or significantly lower expression levels of certain markers characteristic of mature cells. Such markers include CD1, CD3, CD8, CD10, CD13, CD14, CD15, CD19, CD20, CD33, and CD41A.

In addition, at least some endothelial precursor cells also express the AC133 antigen, which was described by Yin et al. in Blood 90, 5002-5112

(1997), Peichev et al., Blood, 2000, 95(3):952-958 and by Miraglia et al. in Blood 90, 5013-5021 (1997). The AC133 antigen is expressed on endothelial and hematopoietic precursor cells, but not on mature cells.

Most, if not all, of the endothelial precursor cells express FLK-1. The
5 CD34 marker is characteristic of precursor cells, such as angioblasts and hematopoietic precursor cells. Approximately 0.5-10% of CD34+ cells are also FLK-1+. For example, approximately 1% of bone marrow cells are CD34+. Of these, approximately 1% are FLK-1+.

High levels of c-kit RNA transcripts are found in primary bone marrow
10 derived mast cells and mast cell lines, while somewhat lower levels are found in melanocytes and erythroid cell lines. Hence c-kit expression is another marker for endothelial precursor cells. The c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor for an unidentified ligand and is a member of the colony stimulating factor-1 (CSF-1)--platelet-derived growth
15 factor (PDGF)--kit receptor subfamily (Besmer et al., (1986) Nature 320, 415-421; Qiu et al., (1988) EMBO J. 7, 1003-1011; Yarden et al., (1987) EMBO J. 6, 3341-3351; Majumder, S., Brown, K., Qiu, F. -H. and Besmer, P. (1988) Mol. Cell. Biol. 8, 4896-4903). c-kit is allelic with the white-spotting (W) locus of the mouse. Mutations at the W locus affect proliferation and/or migration and
20 differentiation of germ cells, pigment cells and distinct cell populations of the hematopoietic system during development and in adult life. The W locus effects hematopoiesis through the erythroid lineages, mast cell lineages and stem cells, resulting in a macrocytic anemia which is lethal for homozygotes of the most severe W alleles, and a complete absence of connective tissue and mucosal mast
25 cells.

A population of endothelial precursor cells can be isolated from mixed cell sources such as bone marrow. The source of cells from which isolated endothelial precursor cells are derived may be any natural or non-natural mixture of cells that contain endothelial precursor cells. The source may be derived from
30 an embryo, or from the post-natal mammal. Preferably, the source of cells is the

hematopoietic microenvironment, such as the circulating peripheral blood, preferably from the mononuclear fraction of peripheral blood, umbilical cord blood, bone marrow, fetal liver, or yolk sac of a mammal. The source of cells therefore need not be embryonic or fetal.

5 Isolated cells are not necessarily pure cells; instead, isolated cells are removed from their natural source, environment or from the mammal where they naturally arose. Isolated cells can also be obtained from in vitro cultures of cell lines or from cultured embryonic cells. Endothelial precursor cells can be purified from a mixed population cells, such as bone marrow cells, by extracting
10 them or removing them from the bone marrow. However, no such purification is needed so long as no adverse immunological reaction will occur upon administration to a mammal. The term purified as applied to the endothelial precursor cell population utilized herein means that the population is significantly enriched in endothelial precursor cells relative to the crude
15 population of cells from which the endothelial precursor cells are isolated.

 Bone-marrow reconstituting hematopoietic stem cells and endothelial cell precursors can be purified, for example, from preparations of bone marrow, fetal liver, circulating blood, or from in vitro-derived cells, such as those derived from allogeneic embryonic cells, nuclear transfer-derived stem cells and
20 parthenogenetically-derived stem cells. Any available method can be used for such purification. Methods that can be employed include, for example, fluorescence-activated cell sorting (FACS) or immunomagnetic separation (for example, see Peichev et al., Blood, 2000, 95(3):952-958); and Otani et al., Nature Medicine, 2002, 8(9): 1004-1010, the contents of both of which are
25 incorporated herein by reference in their entirety). For example, the purification procedure can lead at least to a two-fold, three-fold, five-fold, ten-fold, fifteen-fold, twenty-fold, or twenty-five fold increase in endothelial precursor cells over the total population. The purified population of endothelial precursor cells can contain at least 15%, at least 20%, at least 25%, at least 35%, or at least 50% of
30 endothelial precursor cells.

The methods of the invention can also utilize cellular mixtures comprising 30%, 50%, 75%, 80%, 85%, 90% or 95% of endothelial precursor cells. The methods of the invention can also utilize cell mixtures comprising 99%, 99.9% and even 100% of endothelial precursor cells. Accordingly, cell
5 populations utilized in the invention contain significantly higher levels of endothelial precursor cells than those that exist in nature.

Endothelial precursor cells can be identified by observing their expression patterns or by contacting the cells with a molecule that binds specifically to the extracellular portion of an antigen specific for endothelial
10 precursor cells. The binding of the endothelial precursor cells to the molecule permits the endothelial precursor cells to be sufficiently distinguished from contaminating cells that do not express the antigen to permit identification of the endothelial precursor cells from the contaminating cells.

The cells can also be purified by genetic selection techniques available in
15 the art. For example, a nucleic acid encoding resistance to an antibiotic (such as the neomycin) can be operably linked to a nucleic acid encoding a promoter that is specifically active in an endothelial precursor (such as a KDR promoter) to generate an expression cassette. The expression cassette can then be transfected into embryonic stem cells and the embryonic stem cells can be used to generate
20 endothelial precursor cells that can express the neomycin resistance function. Cells that do not differentiate into endothelial precursor cells will not be resistant to neomycin because the promoter will not be active in those cells.

The molecule used to identify endothelial precursor cells can also be used to separate endothelial precursor cells from the contaminating cells. Such a
25 molecule can be any molecule that is specifically expressed within the endothelial precursor cells or that binds specifically to an antigen that characterizes the endothelial precursor cell. The molecule can be, for example, a monoclonal antibody, a fragment of a monoclonal antibody, or, in the case of an antigen that is a receptor, the ligand of that receptor. For example, in the case of
30 a VEGF receptor, such as FLK-1, the ligand is VEGF. Other molecules that can

be used to identify and separate endothelial precursor cells from other cells include PDGF alpha receptor, VEGF-1 receptor, VEGF-2 receptor, VEGF-3 receptor, VEGF A, VEGF B, VEGF C, VEGF D, VEGF E, EGF, EGF receptor; tumor necrosis factor alpha and tumor necrosis factor receptor, and peptides
5 discovered by phage display to specifically bind to such cells..

Either before or after the crude cell populations are purified as described above, the cells may be further enriched in precursor cells by methods known in the art. For example, human endothelial precursor cells may be pre-purified or post-purified by means of an anti-CD34 antibody, such as the anti-My-10
10 monoclonal antibody described by Civin in U.S. Pat. No. 5,130,144. The hybridoma cell line that expresses the anti-My monoclonal antibody is available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, USA. Some additional sources of antibodies capable of selecting CD34+ cells include AMAC, Westbrook, Me.; Coulter, Hialeah, Fla.; and Becton
15 Dickinson, Mountain View, Calif. CD34+ cells may also be isolated by means of comparable antibodies, which may be produced by methods known in the art, such as those described by Civin in U.S. Pat. No. 5,130,144.

In addition, or as an alternative to, the enrichment with anti-CD34 antibodies, populations of endothelial precursor cells may also be further
20 enriched with the AC133 antibodies described by Yin et al. in Blood 90, 5002-5112 (1997) and by Miraglia et al. in Blood 90, 5013-5021 (1997). The AC133 antibodies may be prepared in accordance with Yin et al., *ibid*, or purchased from Miltenyi Biotec. Hence, the preferred cells of the invention express PDGF B. Such cells may also express FLK-1, CD34, or AC133.

25 Suitable mixtures of cells from a hematopoietic microenvironment may be harvested from a mammalian donor or from an in vitro culture by methods known in the art. For example, precursor endothelial cells may be isolated from bone marrow or from circulating peripheral blood or cells can be differentiated
in vitro from a primitive stem cell. Endothelial precursor cells are mobilized
30 (i.e., recruited) into the circulating peripheral blood by means of cytokines, such

as, for example, G-CSF, GM-CSF, VEGF, SCF (c-kit ligand) and bFGF, chemokines, such as SDF-1, or interleukins, such as interleukins 1 and 8. Hence, endothelial precursor cells can be isolated from blood after recruiting those cells from bone marrow by pre-treatment with one or more of these
5 cytokines. Alternatively, bone marrow may be obtained from a mammal, such as a human patient who will undergo autologous transplantation of the collected cells..

The endothelial precursor cells can be identified within the mixture of cells obtained by exposing the cells to a molecule that binds specifically to the
10 antigen marker characteristic of endothelial precursor cells. The molecule is preferably an antibody or a fragment of an antibody. A convenient antigen marker is PDGF, or a VEGF receptor, for example, a FLK-1 receptor. The cells that express the antigen marker bind to the molecule. The molecule distinguishes the bound cells from unbound cells, permitting separation and isolation. If the
15 bound cells do not internalize the molecule, the molecule may be separated from the cell by methods known in the art. For example, antibodies may be separated from cells with a protease such as chymotrypsin.

The molecule used for isolating the purified populations of endothelial precursor cells is advantageously conjugated with labels that expedite
20 identification and separation. Examples of such labels include magnetic beads, biotin, which may be removed by avidin or streptavidin, fluorochromes, which may be used in connection with a fluorescence-activated cell sorter, and the like.

Any technique may be used for isolation as long as the technique does not unduly harm the endothelial precursor cells. Many such methods are known
25 in the art.

In one embodiment, the molecule is attached to a solid support. Some suitable solid supports include nitrocellulose, agarose beads, polystyrene beads, hollow fiber membranes, and plastic petri dishes. For example, the molecule can be covalently linked to Pharmacia Sepharose 6MB macro beads. The exact
30 conditions and duration of incubation for the solid phase-linked molecules with

the crude cell mixture will depend upon several factors specific to the system employed, as is well known in the art. Cells that are bound to the molecule are removed from the cell suspension by physically separating the solid support from the cell suspension. For example, the unbound cells may be eluted or
5 washed away with physiologic buffer after allowing sufficient time for the solid support to bind the endothelial stem cells.

The bound cells are separated from the solid phase by any appropriate method, depending mainly upon the nature of the solid phase and the molecule. For example, bound cells can be eluted from a plastic petri dish by vigorous
10 agitation. Alternatively, bound cells can be eluted by enzymatically "nicking" or digesting an enzyme-sensitive "spacer" sequence between the solid phase and an antibody. Suitable spacer sequences bound to agarose beads are commercially available, for example, from Pharmacia.

The eluted, enriched fraction of cells may then be washed with a buffer
15 by centrifugation and preserved in a viable state at low temperatures for later use according to conventional technology. The cells may also be used immediately, for example by being infused intravenously into a recipient.

In a desirable variation of the method described above, blood is withdrawn directly from the circulating peripheral blood of a donor. The blood is
20 percolated continuously through a column containing the solid phase-linked molecule to remove endothelial precursor cells. The precursor cell-depleted blood is returned immediately to the donor's circulatory system by methods known in the art, such as hemapheresis. The blood is processed in this way until a sufficient number of precursor cells binds to the column. This method allows
25 rare peripheral blood precursor cells to be harvested from a very large volume of blood, sparing the donor the expense and pain of harvesting bone marrow and the associated risks of anesthesia, analgesia, blood transfusion, and infection.

Other methods for isolating the purified populations of endothelial precursor cells are also known. Such methods include magnetic separation with
30 antibody-coated magnetic beads, and "panning" with an antibody attached to a

solid matrix. Methods for isolating the purified populations of endothelial precursor cells include general fluorescence activated cell sorting (FACS) protocols. In one embodiment, a labeled molecule is bound to the endothelial precursor cells, and the labeled cells are separated by a mechanical cell sorter
5 that detects the presence of the label. The mechanical cell sorter is a fluorescence activated cell sorter (FACS) that is commercially available. Generally, the following FACS protocol is suitable for this procedure:

A Coulter Epics Eliter sorter is sterilized by running 70% ethanol through the systems. The lines are flushed with sterile distilled water.

10 Cells are incubated with a primary antibody diluted in Hank's balanced salt solution supplemented with 1% bovine serum albumin (HB) for 60 minutes on ice. The cells are washed with HB and incubated with a secondary antibody labeled with fluorescein isothiocyanate (FITC) for 30 minutes on ice. The secondary label binds to the primary antibody. The sorting parameters, such as
15 baseline fluorescence, are determined with an irrelevant primary antibody. The final cell concentration is usually set at one million cells per ml.

While the cells are being labeled, a sort matrix is determined using fluorescent beads as a means of aligning the instrument.

Once the appropriate parameters are determined, the cells are sorted and
20 collected in sterile tubes containing medium supplemented with fetal bovine serum and antibiotics, usually penicillin, streptomycin and/or gentamicin. After sorting, the cells are re-analyzed on the FACS to determine the purity of the sort.

In another embodiment, the invention is directed to isolated populations of precursor cells that express a suitable marker, for example, PDGF B or a
25 VEGF receptor, such as, for example, the FLK-1 receptor. This embodiment further includes isolation of purified populations of such cells. The PDGF B+ precursor cells include, for example, endothelial precursor cells. The source of cells from which the precursor cells are obtained include both pre-natal and post-natal sources. Post-natal sources are preferred.

30

Generating Endothelial Precursor Cells

In addition to providing methods for isolating endothelial precursor cells, the invention provides methods for producing such cells. Hematopoietic stem cells (HSCs) and endothelial cell precursors (ECPs) produced in this manner can be genetically modified to express a useful gene product, for example, a gene product that augments repair of vascular injury or disease, or a gene product that prevents development of vascular disease. The hematopoietic stem cells and endothelial cell precursors can home to vascular tissues and provide angiogenesis (for example, in the coronary arteries of the heart), thereby restoring vascular tissues that have been injured or have become diseased.

In one embodiment of the present invention, hematopoietic stem cells and endothelial cell precursors are isolated from a human or a non-human mammal by available methods, for example, as described above in the previous section. These cells can be genetically modified in vitro to contain a genomically integrated DNA expression construct encoding a gene that confers therapeutic effect when it is expressed by endothelial cells in the heart or arteries affected with a vascular disease such as atherosclerosis.

In an alternative embodiment of the invention, healthy somatic cells are isolated from a human or a non-human mammal and used for generating totipotent or pluripotent embryo-derived stem cells (e.g., embryonic stem cells). In this embodiment, the nucleic from these somatic cells are inserted into an enucleated oocyte by available procedures to generate a nuclear transfer unit that is stimulated to divide, thereby generating totipotent or pluripotent embryo-derived stem cells. The totipotent or pluripotent embryo-derived stem cells can be induced to differentiate into hematopoietic stem cells, which in turn can differentiate to generate genetically modified endothelial cell precursors of the invention. Prior to nuclear transfer, the somatic cell can be genetically modified to contain a gene that confers a therapeutic effect when expressed by endothelial cells, or alternatively, such modifications can be introduced in the resulting stem cells.

All types of somatic cells can be utilized as donor cells for this purpose. For example, the donor cell or donor cell nucleus can be selected from the group consisting of epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes, erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, muscle cells, skin
5 cells, lung cells, pancreatic cells, liver cells, stomach cells, intestinal cells, heart cells, bladder cells, reproductive organ cells, urethra cell, and kidney cells.

The hematopoietic stem cells and endothelial cell precursors, whether genetically modified or not, are then administered to a patient with a vascular
10 disease, whereupon the hematopoietic stem cells or endothelial cell precursors home to sites of vascular injury or areas of ischemic injury (see, for example, Asahara et al., 1997, "Isolation of putative progenitor endothelial cells for angiogenesis," Science 275: 964-967, the contents of which are incorporated herein by reference). After reaching the site of vascular injury the hematopoietic
15 stem cells and/or endothelial cell precursors help to prevent or repair vascular disease or vascular injury. Expression of a transgene can further enhance the therapeutic effect of these cells.

Advanced Cell Technology, Inc. and other groups have developed methods for transferring the genetic information in the nucleus of a somatic or
20 germ cell from a child or adult into an unfertilized egg cell, and culturing the resulting cell to divide and form a blastocyst embryo having the genotype of the somatic or germ nuclear donor cell. Methods for cloning by such methods are referred to as cloning by "somatic cell nuclear transfer," because somatic donor cells are commonly used. Methods for cloning by nuclear transfer are available,
25 and are described, for example, in U.S. Patent Nos. 6,235,970 (Stice et al.) and 6,147,276 (Campbell et al.), and in U.S. Patent Nos. 5,994,619 and 6,235,969 of Stice et al., the contents of all three are incorporated herein by reference in their entirety.

Methods for human therapeutic cloning have been described. For
30 example, methods that use nuclear transfer cloning to produce cells and tissues

for transplant therapies that are histocompatible with the transplant recipient are described in U.S. Application No. 09/797,684 filed March 5, 2001. This application also discloses assay methods for determining the immune-compatibility of cells and tissues for transplant the contents of which are

5 incorporated herein by reference in their entirety. Similar methods are also described in U.S. Application No. 10/227,282 ("Screening Assays for Identifying Differentiation-Inducing Agents and Production of Differentiated Cells for Cell Therapy"), filed August 26, 2002, the contents of which are also incorporated herein by reference in their entirety, which further discloses

10 screening methods that make use of gene trapped cell lines and provide means for efficiently identifying combinations of biological, biochemical, and physical agents or conditions that induce stem cells to differentiate into cell types useful for transplant therapy. Methods for producing totipotent and pluripotent stem cells are also described in U.S. Application No. 09/995,659 filed November 29,

15 2001, and International Application No. PCT/US02/22857 filed July 18, 2002, which further describe methods for producing histocompatible cells and tissues for transplant by androgenesis and gynogenesis; and in U.S. Application No. 09/520,879 filed April 5, 2000, which discloses methods for producing "rejuvenated" or "hyper-young" cells having increased proliferative potential

20 relative to cells of the donor animal. A method for obtaining totipotent and pluripotent stem cells from embryos generated by parthenogenesis is also reported by Cibelli et al., who describe the isolation of a non-human primate stem cell line from the inner cell mass of parthenogenetic Cynomologous monkey embryos that is capable of differentiating into cell types of all three

25 embryonic germ layers (see Science (2002) 295:819, the contents of which are incorporated herein by reference in their entirety.) The disclosures of all of the above-listed patent applications are also incorporated herein by reference in their entirety.

A general procedure for cloning by fusion of a somatic cell is provided

30 below. The procedure is meant to be exemplary. Many variations and

modifications can be made to such a procedure by one of skill in the art without deviating from the invention.

In general, oocytes are isolated from the ovaries or reproductive tract of a human or non-human mammal, matured in vitro, and stripped of cumulus cells to prepare for nuclear transfer. Removal of the endogenous chromosomes of the oocyte is referred to as "enucleation." Enucleation of the recipient oocyte is performed after the oocyte has attained the metaphase II stage, and can be carried out before or after nuclear transfer. Enucleation can be confirmed by visualizing chromosomal DNA in TL-HEPES medium plus Hoechst 33342 (3 $\mu\text{g/ml}$; Sigma).

Individual donor cells are placed in the perivitelline space of the recipient enucleated oocyte, and the oocyte and donor cell are fused together to form a single cell (nuclear transfer unit) e.g., by electrofusion. The nuclear transfer units are activated, and are incubated in suitable medium under conditions that promote growth of the nuclear transfer unit. During this period of incubation, the nuclear transfer units can be transferred to culture plates containing a confluent feeder layer. Feeder layers of various cell types from various species, e.g., irradiated mouse embryonic fibroblasts, that are suitable for the invention are described, for example, in U.S. Patent No. 5,945,577, the contents of which are incorporated herein by reference in their entirety.

Genetically modified nuclei can be generated and fused with enucleated oocytes as follows. Primary cultures of somatic cells are isolated and grown in vitro using available methods. Such methods are described, for example, in U.S. Patent No. 6,011,197 (Strelchenko et al.), and in U.S. Patent No. 5,945,577 (Stice et al.), the contents of both of which are incorporated herein by reference in their entirety.

The somatic donor cell used for nuclear transfer to produce a nuclear transplant unit or embryo according to the present invention can be of any germ cell or somatic cell type in the body. For example, the donor cell can be a germ cell, or a somatic cell selected from the group consisting of fibroblasts, B cells, T

cells, dendritic cells, keratinocytes, adipose cells, epithelial cells, epidermal cells, chondrocytes, cumulus cells, neural cells, glial cells, astrocytes, cardiac cells, esophageal cells, muscle cells, melanocytes, hematopoietic cells, macrophages, monocytes, and mononuclear cells. The donor cell can be
5 obtained from any organ or tissue in the body; for example, it can be a cell from an organ selected from the group consisting of liver, stomach, intestines, lung, stomach, intestines, lung, pancreas, cornea, skin, gallbladder, ovary, testes, vasculature, brain, kidneys, urethra, bladder, and heart, or any other organ.

A general procedure for isolating primary cultures of fibroblast cells is as
10 follows: Minced tissue is incubated overnight at 10 °C. in trypsin, cells are washed and then are plated in tissue culture dishes and cultured in alpha-MEM medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, Utah), penicillin (100 IU/ml) and streptomycin (50 µl/ml). The fibroblast cells can be isolated at virtually any time in
15 development, ranging from approximately post embryonic disc stage through adult life of the animal (for example, for bovine, from day 12 to 15 after fertilization to 10 to 15 years of age).

A general procedure for stably introducing a genetic expression construct into the genomic DNA of the cultured fibroblasts by electroporation is described
20 below. Other available transfection methods, such as microinjection or lipofection can also be used to introduce heterologous DNA into the cells.

Culture plates containing propagating fibroblast cells are incubated in trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, N.Y.) until the cells are in a single cell suspension. The cells are spun down at
25 500xg and re-suspended at a density of about 5 million cells per ml with phosphate buffered saline (PBS). A vector or nucleic acid construct containing the an expression cassette encoding the gene product of interest is added to the cells in the electroporation chamber . After providing a standard electroporation pulse, the fibroblast cells are transferred back into the growth medium (alpha-
30 MEM medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal

calf serum (FCS) (Hyclone, Logan, Utah), penicillin (100 IU/ml) and streptomycin (50 μ l/ml)).

The day after electroporation, attached fibroblast cells are selected for stable integration of the vector or nucleic acid construct by culturing them for up to 15 days in growth medium containing a selective agent that will select for cells having the vector or nucleic acid construct. At the end of the selection period, colonies of stable transgenic cells are present. Each colony is propagated independently of the others. Transgenic fibroblast cells can be further tested for expression of the gene product of interest, and genomic integration of the expression construct can be confirmed by available methods; e.g., by PCR amplification and analysis by agarose gel electrophoresis.

Stably transfected fibroblast cells are used as nuclear donors in the nuclear transfer (NT) procedure. Procedures for cloning by nuclear transfer are available in the art. For example, methods for cloning by somatic cell nuclear transfer are described in detail in U.S. Patent No. 6,147,276 (Campbell et al.), and in co-owned and co-assigned U.S. Patent Nos. 5,945,577 and 6,235,969 of Stice et al.

In general, oocytes are isolated from the ovaries or reproductive tract of a human or non-human mammal and are matured in vitro. The oocytes are stripped of cumulus cells to prepare for nuclear transfer. Enucleation of the recipient oocyte is performed after the oocyte has attained the metaphase II stage, and can be carried out before or after nuclear transfer. Individual donor cells (fibroblasts) are then placed in the perivitelline space of the recipient oocyte, and the oocyte and donor cell are fused together to form a single cell (an nuclear transfer unit) using electrofusion techniques; e.g., by applying a single one fusion pulse consisting of 120 V for 15 μ sec to the nuclear transfer unit in a 500 μ m gap chamber. The nuclear transfer units are then incubated in suitable medium.

A variety of different procedures for artificially activating oocytes are available and have been described. See U.S. Application No. 09/467,076

(Cibelli et al.), filed December 20, 1999, the contents of which are incorporated herein by reference in their entirety. Following activation, the nuclear transfer units are washed and cultured under conditions that promote growth of the nuclear transfer unit to have from 2 to about 400 cells. During this time, the nuclear transfer units can be transferred to well plates containing a confluent feeder layer; e.g., a feeder layer of mouse embryonic fibroblasts. Feeder layers of various cell types from various species that are suitable for the invention are described, for example, in U.S. Patent No. 5,945,577. Multicellular non-human nuclear transfer units produced in this manner can be transferred into recipient non-human females of the same species as the donor nucleus and recipient oocyte, for development into transgenic non-human mammals. Alternatively, the nuclear transfer units can be incubated until they reach the blastocyst stage, and the inner cell mass (ICM) cells of these nuclear transfer units can be isolated and cultured in the presence or absence of a feeder layer to generate pluripotent or totipotent embryonic stem cells. These stem cells can then be differentiated to generate downstream cultured stem cells such as the mesodermal precursors to hemangioblasts.

Multicellular non-human nuclear transfer units produced in this manner can be transferred as embryos into recipient non-human females of the same species as the donor nucleus and recipient oocyte, for development into transgenic non-human mammals. Alternatively, the nuclear transfer units can be incubated in vitro until they reach the blastocyst stage, and the inner cell mass (ICM) cells of these nuclear transfer units can be isolated and cultured in the presence or absence of a feeder layer to generate pluripotent or totipotent embryo-derived stem cells, including totipotent embryonic stem cells.

Cellular Differentiation

Methods are available for isolating cells within the inner cell mass of a blastocyst produced by nuclear transfer, and culturing these to generate pluripotent and totipotent embryo-derived cell lines, including totipotent

embryonic stem cell lines. For example, see U.S. Patent Nos. 5,905,042 and 5,994,619 of Stice et al., the contents of both of which are incorporated herein by reference. Using available methods, totipotent and pluripotent stem cells derived from nuclear transfer-generated blastocysts, e.g., embryonic stem cells, can be cultured under conditions that direct or allow differentiation into a variety of partially and fully differentiated somatic cell types, including hematopoietic stem cells. For example, see Wakayama et al., "Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer, 2001, Science, 292:740-3; Talbot et al., "Spontaneous differentiation of porcine and bovine embryonic stem cells (epiblast) into astrocytes or neurons," 2002, In Vitro Cell Dev Biol Anim., 38(4):191-7; and Mitalipova et al., "Pluripotency of bovine embryonic cell line derived from precompacting embryos," 2001, Cloning, 3(2):59-67, the contents of all three of which are incorporated herein by reference. Methods for inducing the differentiation of pluripotent human blastocyst-derived embryonic stem cells into hematopoietic stem cells are also available (U.S. Patent No. 6,280,718, Kaufman et al., "Hematopoietic differentiation of human pluripotent embryonic stem cells," the contents of which are incorporated herein by reference).

Accordingly, stem cells isolated or generated as described herein can readily be differentiated into endothelial precursor cells and hematopoietic stem cells.

Moreover, the invention provides methods for rejuvenating senescent cells that ordinarily would not be able to differentiate into other cell types, so that those senescent cells can give rise to therapeutically active cells. In particular, the invention provides a method of generating cardiac myocytes from senescent bone marrow cells that could not otherwise give rise to such cardiac myocytes. Such a method involves obtaining bone marrow cells, contacting the bone marrow cells with platelet derived growth factor AB and thereby generating cardiac myocyte from the bone marrow cells. As discussed, the bone marrow cells can be senescent cells and need not be obtained from embryonic

tissues. Instead, the bone marrow cells can be obtained from an older patient, even one with a vascular disease, so that after re-introducing the cells to the patient no tissue rejection or other immunological problems will arise. Hence, the inventive methods avoid side effects and other complications.

5 To promote cardiac myocytes formation from bone marrow cells, the bone marrow cells can be cultured in a sufficient amount of platelet derived growth factor AB for a time and under conditions sufficient to generate myocytes. Platelet derived growth factor is commercially available and can be obtained, for example, from R&D Systems.

10 A sufficient amount of platelet derived growth factor AB is about 0.001 ng/mL to about 10 mg/mL, or about 0.01 ng/mL to about 1 mg/mL, or about 0.1 ng/mL to about 100 ng/mL or about 1 ng/mL to about 100 ng/mL platelet derived growth factor AB. In certain embodiments, senescent bone marrow cells were successfully treated with platelet derived growth factor AB at
15 concentrations of about 10 ng/mL and 100 ng/mL.

 The time used to generate cardiac myocytes from bone marrow by PDGF AB treatment can vary. For example, culturing bone marrow cells in the presence of PDGF AB for a time period of a few days (about 3 days) to several weeks (about 5 weeks) can lead to cardiac myocytes generation from bone
20 marrow cells. In experiments described herein, bone marrow cells were successfully cultured for about 1 week in order to facilitate cardiac myocyte formation.

 Conditions required for culturing bone marrow cells to generate cardiac myocytes comprise the conditions normally employed for culturing mammalian
25 cells *in vitro*. Inclusion of vascular endothelial growth factor (VEGF, at about 10ng/mL), fibroblast growth factor-2 (FGF-2) (at about 5 ng/mL) and heparin (at about 50µg/mL) also helps support the generation of cardiac myocytes from bone marrow cells *in vitro*.

30

Syngeneic hematopoietic stem cells and endothelial cell precursors

In a useful embodiment of the invention, bone marrow or somatic cells are taken from a patient with a vascular disease. These syngeneic cells can be treated to generate useful cells for treatment of vascular diseases. For example, bone marrow cells can be cultured with platelet derived growth factor AB to generate syngeneic cardiac myocytes that can be re-administered to the patient. Such bone marrow and other somatic cells can also be genetically modified to contain a gene that confers a therapeutic effect. While the genetically modified bone marrow cells that then be administered, the genetically-modified somatic cells are cloned by somatic cell nuclear transfer to produce pluripotent embryo-derived stem cells. Such syngeneic stem cells can be induced to differentiate into hematopoietic stem cells and endothelial cell precursors that can give rise to genetically modified endothelial cells *in vivo*. The genetically modified hematopoietic stem cells and endothelial cell precursors are then administered to a patient as an autologous transplant, whereupon the endothelial cells derived therefrom home to sites of cardiac angiogenesis or vessel repair. Since the transplanted bone marrow cells, hematopoietic stem cells and endothelial cell precursors are syngeneic with the patient, they are histocompatible and do not elicit an immune response, unless such a response is elicited by expression of the transgene.

An alternative embodiment of the invention that does not use nuclear transfer-derived cells can be practiced as follows:

Endothelial cell precursors can also be isolated from the patient, genetically modified *in vitro* to contain a gene that confers a therapeutic effect, and are reintroduced to the patient as described in PCT Publication WO 99/37751 by Shahin Rafil, Larry White and Malcolm A. Moore, and U.S. Patent No. 5,980,887 (Isner et al.), the contents of which are incorporated herein by reference in their entirety. In brief, a sample of blood is drawn from the patient, typically 50 – 200 ml. Prior to venipuncture, the patient can be treated with factors such as Granulocyte Colony Stimulating Factor (GCSF), which

stimulates an increase in the number of circulating endothelial cell precursors. The leukocyte fraction is separated by Ficoll density gradient, then plated briefly to remove adhesive cells. A population of cells positive for antigens specific for endothelial cell precursors, including but not limited to CD34, VEGFR-2, and AC133, is then isolated. For example, the remaining cells can be treated with fluorochrome labeled antibodies to the antigens specific for endothelial cell precursors and isolated by Fluorescence Activated Cell Sorting (FACS). Alternatively, endothelial cell precursors can be isolated by magnetic beads coated with the above antibodies to the above antigens, as is available in the art. Once purified, the population of endothelial cell precursors are cultured in vitro in suitable medium (e.g., M199 media supplemented with 20% fetal bovine serum), and the cells are genetically modified using methods known in the art. Following genetic modification, the endothelial cell precursors are intravenously reintroduced to the patient.

Allogeneic, HLA-matched endothelial cell precursors

Banks of bone marrow cells or of pre-made embryonic stem cell lines can be isolated, where the bone marrow cells or embryonic stem cell lines are each homozygous for at least one MHC gene. Such banks of cells serve as an alternative to using nuclear transfer cloning to produce syngeneic embryonic stem cells de novo and inducing these to differentiate into the required cells for every patient that is in need of therapeutic transplant. However, homozygous embryos generated in vitro or vivo can serve as a source of homozygous MHC stem cells.

The MHC genes of humans are also referred to as HLA (human leukocyte antigen) genes or alleles. Such MHC and HLA genes are highly polymorphic, and banks of different embryonic stem cell lines and different bone marrow isolates with different MHC and HLA genes will include a large number of different embryonic stem cell lines. Once such banks of bone marrow isolates or embryonic stem cells with homozygous MHC alleles are produced, it is

possible to provide a patient in need of cell transplant with MHC-matched cells and tissues by selecting and/or expanding a line of bone marrow cells of embryonic stem cells that has MHC allele(s) that match one of those of the patient. The bone marrow or embryonic stem cells can be treated with PDGF
5 AB or other agents to differentiate into the type of cells that the patient requires. Methods for preparing a bank of embryonic stem cell lines that are homozygous for the MHC alleles, and for using these to provide MHC-matched cells and tissues for transplantation therapies are described in co-pending U.S. Provisional Patent Application No. 60/382,616, entitled, " A Bank of Nuclear Transfer-
10 Generated Stem Cells for Transplantation Having Homozygous MHC Alleles, and Methods for Making and Using Such a Stem Cell Bank, filed May 24, 2002, the disclosure of which is incorporated herein by reference in its entirety.

Therefore, in another useful embodiment of the invention, the bone marrow and nuclear donor cells that are genetically modified are not obtained
15 from the patient. Instead, they are taken from a person who has HLA alleles that match those of the patient. More simply, the bone marrow or nuclear donor cells are taken from a person who has homozygous HLA alleles that match at least one HLA allele of the patient. A bank of samples of viable nuclear donor cells, each sample made up of cells having homozygous HLA alleles that match
20 an HLA allele found in the population, is prepared and maintained for practicing this embodiment. See U.S. Provisional Patent Application No. 60/382,616. As described above for syngeneic transplant therapy, genetically modified, HLA-matched hematopoietic stem cells and endothelial cell precursors produced by the invention are administered to a patient as a heterologous transplant, to give
25 rise to endothelial cells that home to and incorporate into the tumor vasculature to disrupt or inhibit tumor angiogenesis. Since the transplanted hematopoietic stem cells and endothelial cell precursors are HLA-matched to the patient, they are partially histocompatible with the patient, and so do not elicit the strong rejection response that would be elicited by a completely allogeneic transplant.

In an alternative embodiment, cells of one or more of the established human embryonic stem cell lines are genetically modified, and available methods are used to induce the genetically modified embryonic stem cells to differentiate into hematopoietic stem cells and endothelial cell precursors. These
5 hematopoietic stem cells and endothelial cell precursors can then give rise to genetically modified endothelial cells that confer a therapeutic effect when recruited into a sites of vascular injury or ischemic myocardium. Alternatively, hematopoietic stem cells and endothelial cell precursors can be isolated directly from a young person other than the patient and when appropriate to the needs of
10 that patient, genetically modified, to confer a therapeutic effect. The hematopoietic stem cells and endothelial cell precursors obtained from differentiating embryonic stem cells or directly from a person other than the patient can then be transplanted into the patient.

15 Genetic modification of somatic cells, stem cells and endothelial cell precursors

Transgenic cells of the invention that are genetically modified to contain a stably integrated gene that is expressed in endothelial cells and that confers a therapeutic effect are obtained by methods available in the art. Recombinant
20 expression vectors are made and introduced into the cells using standard techniques, e.g., electroporation, lipid-mediated transfection, or calcium-phosphate mediated transfection, and cells containing stably integrated expression constructs are selected or otherwise identified, also using standard techniques known in the art. Methods for making recombinant DNA expression
25 constructs, introducing them into eukaryotic cells, and identifying cells in which the expression construct is stably integrated and efficiently expressed, are described, for example, in Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press (1989); Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2d Edition, Cold Spring Harbor

Laboratory Press (2001). Such methods useful for practicing the present invention are also described, for example, in U.S. Patent No. 5,980,887.

A variety of different types of genes that confer a therapeutic effect when expressed in endothelial cells in sites of vascular injury or I-ischemic myocardium. For examples, endothelial precursor cells of the invention can be used to administer therapeutic agents such as enzymes, peptides and/or proteins with biological activity, nucleic acids or genes that encode therapeutic polypeptides, expression vectors or other nucleic acid constructs, for example, naked plasmid DNAs, any vector carrying one or more genes, any sense or anti-sense RNA, or any ribozyme. Nucleic acids encoding such therapeutic agents are introduced into endothelial precursor cells based upon their ability to optimally treat one or more vascular conditions. For example, the endothelial precursor cell can be designed to help control, diminish or otherwise facilitate improved arterial blood flow in the region of the atherosclerotic lesion.

Such therapeutic agents include, for example, thrombolytic agents such as streptokinase, tissue plasminogen activator, plasmin and urokinase, anti-thrombotic agents such as tissue factor protease inhibitors (TFPI), anti-inflammatory agents, metalloproteinase inhibitors, nematode-extracted anticoagulant proteins (NAPs) and the like. Other examples of therapeutic agents that can be expressed in the endothelial precursor cells of the invention include the following: agents that modulate lipid levels (for example, HMG-CoA reductase inhibitors, thyromimetics, fibrates, agonists of peroxisome proliferator-activated receptors (PPAR) (including PPAR-alpha, PPAR-gamma and/or PPAR-delta)); agents that modulate oxidative processes such as modifiers of reactive oxygen species; agents that modulate insulin resistance or glucose metabolism (e.g. agonists of PPAR-alpha, PPAR-gamma and/or PPAR-delta, modifiers of DPP-IV, and modifiers of glucocorticoid receptors); agents that modulate expression of receptors or adhesion molecules or integrins on endothelial cells or smooth muscle cells in any vascular location; agents that modulate the activity of endothelial cells or smooth muscle cells in any vascular

location; agents that modulate inflammation associated receptors (e.g. chemokine receptors, RAGE, toll-like receptors, angiotensin receptors, TGF receptors, interleukin receptors, TNF receptors, C-reactive protein receptors, and other receptors involved in inflammatory signaling pathways including the
5 activation of NF-kb); agents that modulate proliferation, apoptosis or necrosis of endothelial cells, vascular smooth muscle, lymphocytes, monocytes, and neutrophils that adhere to or within the vessel; agents that modulate production, degradation, or cross-linking of any extracellular matrix proteins (e.g. collagen, elastin, and proteoglycans); agents that modulate activation, secretion or lipid
10 loading of any cell type within mammalian vessels; agents that modulate the activation or proliferation of dendritic cells within mammalian vessels; and agents that modulate the activation or adhesion of platelets within blood vessels.

The endothelial precursor cells utilized in the methods of the invention express, or over-express, platelet-derived growth factor ("PDGF"). In some
15 embodiments, the endothelial precursor cells are genetically modified to have a recombinant or transgenic PDGF DNA, for example, a PDGF DNA operably linked to a promoter useful for over-expression of a PDGF gene product.

Naturally occurring, platelet-derived growth factor is a disulfide-bonded dimer having two polypeptide chains, namely the "A" and "B" chains, with the A
20 chain being approximately 60% homologous to the B chain. Naturally occurring PDGF is found in three dimeric forms, namely PDGF-AB heterodimer, PDGF-BB homodimer, or PDGF-AA homodimer. Hannink et al., Mol. Cell. Biol., 6, 1304-1314 (1986). PDGF-AB has been identified as a predominate naturally occurring form. However, some data indicates that the PDGF-BB homodimer
25 may be effective for wound healing. Each monomeric subunit of the biologically active dimer, irrespective of whether it is an A chain monomer or a B chain monomer, contains eight cysteine residues. Some of these cysteine residues form interchain disulfide bonds that hold the dimer together. As used
30 herein, the term PDGF means any PDGF polypeptide or protein, including PDGF A, PDGF B, PDGF AB, PDGF BB, and PDGF AA.

The A polypeptide of human PDGF can be any mammalian PDGF A polypeptide including, for example, human, mouse, rat, rabbit, goat, bovine, horse, sheep and any other mammalian PDGF A polypeptide. The following sequence is one example of an amino acid sequence of a human PDGF A

5 polypeptide (SEQ ID NO:1):

1 MRTWACL L L L L GCGYLAHALA EEAEIPRELI ERLARSQIHS
 41 IRDLQRLLEI DSVGAEDALE TNLRAHGSHT VKHVPEKRPV
 81 PIRRKRSIEE AIPAVCKTRT VIYEIPRSQV DPTSANFLIW
 121 PPCVEVKRCT GCCNTSSVKC QPSRVHHRVS KVAKVEYVRK
 10 161 KPKLKEVQVR LEEHLECACA TSNLNPDHRE EETGRRRESG
 201 KKRK

A nucleic acid that encodes a human PDGF A polypeptide can be found in the NCBI database at accession number X03795, gi:35365. See website at www.ncbi.nlm.nih.gov.

15 The following sequence is an example of a mouse PDGF A sequence (SEQ ID NO:2).

1 MRTWACL L L L L GCGYLAHALA EEAEIPRELI ERLARSQIHS
 41 IRDLQRLLEI DSVGAEDALE TSLRAHGSHA INHVPEKRPV
 81 PIRRKRSIEE AVPAVCKTRT VIYEIPRSQV DPTSANFLIW
 20 121 PPCVEVKRCT GCCNTSSVKC QPSRVHHRVS KVAKVEYVRK
 161 KPKLKEVQVR LEEDLECACA TSNLNPDHRE EETDVR

A nucleic acid that encodes a mouse PDGF A polypeptide can be found in the NCBI database at accession number NM 008808, gi:6715565. See website at www.ncbi.nlm.nih.gov.

25 Other sequences for PDGF A can readily be obtained by one of skill in the art, for example, in the GenBank database of sequences. Variability in these and other sequences is permitted so long as the PDGF A polypeptide can dimerize with PDGF B and/or function in cell-to-cell communication.

30 The PDGF B polypeptide found in human platelets has been identified as a 109 amino acid cleavage product (PDGF-B₁₀₉) of a 241 amino acid precursor

polypeptide Johnsson et al., EMBO Journal, 3(5), 921-928 (1984). An example of a human sequence for the PDGF B polypeptide is provided below (SEQ ID NO:3).

```

1  MNRCWALFLS LCCYLRLVSA EGDPIPEELY EMLSDHSIRS
5  41  FDDLQRL LHG DPGEEDGAEL DLNMTRSHSG GELESLARGR
    82  RSLGSLTIAE PAMIAECKTR TEVFEISRRL IDRTNANFLV
121 WPPCVEVQRC SGCCNNRNVQ CRPTQVQLRP VQVRKIEIVR
161 KKPIFKKATV TLEDHLACKC ETVAARPVT RSPGGSQEQR
201 AKTPQTRVTI RTVRVRRPPK GKHRKFKHTH DKTALKETLG
10 241  A

```

A nucleic acid that encodes a human PDGF B polypeptide can be found in the NCBI database at accession number X02811, gi:35371. See website at www.ncbi.nih.nlm.gov.

The following sequence is an example of a mouse PDGF B sequence (SEQ ID NO:4).

```

1  MNRCWALFLP LCCYLRLVSA EGDPIPEELY EMLSDHSIRS
    41  FDDLQRL LHR DSVDEGAEL DLNMTRAHSG VELESSSRGR
    81  RSLGSLAAAE PAVIAECKTR TEVFQISRNL IDRTNANFLV
121 WPPCVEVQRC SGCCNNRNVQ CRASQVQMRP VQVRKIEIVR
20 161 KKPIFKKATV TLEDHLACKC ETIVTPRPVT RSPGTSREQR
    201 AKTPQARVTI RTVRIRPPK GKHRKFKHTH DKAALKETLG
    241  A

```

A nucleic acid that encodes a mouse PDGF B polypeptide can be found in the NCBI database at accession number NM 011057, gi:6755009. See website at www.ncbi.nih.nlm.gov.

As recognized by one of skill in the art, these PDGF polypeptides from different mammalian species have similar amino acid sequences. According to the invention any PDGF polypeptide from any mammalian species can be utilized in the practice of the invention so long as the PDGF polypeptide can stimulate endothelial cells to promote angiogenesis.

A 109 amino acid PDGF B polypeptide is believed to be the mature form of PDGF in humans and constitutes a cleavage product of the PDGF-B precursor protein. Homology with the precursor protein begins at amino acid 82 of the 241 amino acid precursor protein and continues for 109 amino acids yielding, for example, a polypeptide with the following sequence (SEQ ID NO:5):

```

5   82  RSLGSLTIAE PAMIAECKTR TEVFEISRRL IDRTNANFLV
      121 WPPCVEVQRC SGCCNNRNVQ CRPTQVQLRP VQVRKIEIVR
      161 KKPIFKKATV TLEDHLACKC ETVAAARPVT RSPGGSQEQR
      201 AKTPQTRVTI RTVRVRPPK GKHRKFKHTH DKTALKETLG
10  241  A

```

Another form of PDGF-B (PDGF-B₁₁₉), corresponds to the first 119 amino acids of the PDGF-B precursor protein (SEQ ID NO:6):

```

      1  MNRCWALFLS LCCYLRLVSA EGDPIPEELY EMLSDHSIRS
      41  FDDLQRLHGG DPGEEDGAEL DLNMTRSHSG GELESLARGR
15   82  RSLGSLTIAE PAMIAECKTR TEVFEISRRL IDRTNANFL

```

This PDGF-B₁₁₉ form has also been identified as a major cleavage product of the precursor protein when the entire gene is encoded into a transfected mammalian host. See U.S Pat. No. 5,149,792.

Human platelet-derived growth factor is believed to be the major mitogenic growth factor in serum for connective tissue cells. PDGF can positively affect mitogenesis in arterial smooth muscle cells, fibroblast cell lines, and glial cells. Deuel et al., J. Biol. Chem., 256(17), 8896-8899 (1981). See also, e.g., Heldin et al., J. Cell Physiol., 105, 235 (1980) (brain glial cells); Raines and Ross, J. Biol. Chem., 257, 5154 (1982) (monkey arterial smooth muscle cells).

In some embodiments, the endothelial precursor cells are genetically modified to have a recombinant or transgenic PDGF receptor DNA, for example, a PDGF receptor DNA operably linked to a promoter useful for over-expression of a PDGF receptor. Examples of amino acid and nucleotide sequences for the PDGF receptor(s) can be found in the NCBI database. See website at

www.ncbi.nih.nlm.gov. One example of a sequence for the human PDGF alpha receptor is provided below (accession number PFHUGA, gi:66814, SEQ ID NO:35):

```

5      1  MGTSHPAFLV  LGCLLTGLSL  ILCQLSLPSI  LPNENEKVVO
      41  LNSSFSLRCF  GESEVSWQYP  MSEEESDVE  IRNEENNSGL
      81  FVTVLEVSSA  SAAHTGLYTC  YYNHTQTEEN  ELEGRHIYIY
     121  VDPDPVAFVP  LGMTDYLIV  EDDDSAIIPC  RTTDPETPVT
     161  LHNSEGVVPA  SYDSRQGFNG  TFTVGPYICE  ATVKGKKFQT
     201  IPFNVYALKA  TSELDLEMEA  LKTVYKSGET  IVVTCAVFNN
10     241  EVVDLQWTYP  GEVKGKGITM  LEEIKVPSIK  LVYTLTVPEA
     281  TVKDSGDYEC  AARQATREVK  EMKKVTISVH  EKGFIIEIKPT
     321  FSQLEAVNLH  EVKHFVVEVR  AYPPPRISWL  KNNLTLIENL
     361  TEITTDVEKI  QEIRYRSKLK  LIRAKEEDSG  HYTIVAQNED
     401  AVKSYTFELL  TQVPSSILD  VDDHHGSTGG  QTVRCTAEGT
15     441  PLPDIEWMIC  KDIKKCNNET  SWTILANNVS  NIITEIHSRD
     481  RSTVEGRVTF  AKVEETIAVR  CLAKNLLGAE  NRELKLVAPT
     521  LRSELTVAAG  VLVLLVIVII  SLIVLVVIWK  QKPRYEIRWR
     561  VIESISPDGH  EYIYVDPMQL  PYDSRWEFPR  DGLVLGRVLG
     601  SGAFGKVVEG  TAYGLSRSQP  VMKVAVKMLK  PTARSSEKQA
20     641  LMSELKIMTH  LGPHLNIVNL  LGACTKSGPI  YIITEYCFYG
     681  DLVNYLHKNR  DSFLSHHPEK  PKKELDIFGL  NPADESTRSY
     721  VILSFENNGD  YMDMKQADTT  QYVPMLERKE  VSKYSDIQRS
     761  LYDRPASYKK  KSMLDSEVKN  LLSDDNSEGL  TLLDLLSFTY
     801  QVARGMEFLA  SKNCVHRDLA  ARNVLLAQGK  IVKICDFGLA
25     841  RDIMHDSNYV  SKGSTFLPVK  WMAPESIFDN  LYTTLSDVWS
     881  YGILLWEIFS  LGGTPYPGMM  VDSTFYNKIK  SGYRMAKPDH
     921  ATSEVYEIMV  KCWNSEPEKR  PSFYHLSEIV  ENLLPGQYKK
     961  SYEKIHLDFL  KSDHPAVARM  RVDSDNAYIG  VTYKNEEDKL
    1001  KDWEGGLDEQ  RLSADSGYII  PLPDIDPVPE  EEDLGKRNHR
30    1041  SSQTSEESAI  ETGSSSSTFI  KREDETIEDI  DMMDDIGIDS
    1081  SDLVEDSFL

```

An example of a sequence for the human PDGF beta receptor is provided below (accession number NP 002600, gi:4505683, SEQ ID NO:36):

```

35      1  MRLPGAMPAL  ALKGELLLLS  LLLLLLEPQIS  QGLVVTPPGP
      41  ELVLNVSTF  VLTCSGSAPV  VWERMSQEPP  QEMAKAQDGT
      81  FSSVLTLTNL  TGLDTGEYFC  THNDSRGLET  DERKRLYIFV
     121  PDPTVGFLPN  DAEELFIFLT  EITEITIPCR  VTDLPQLVVTL
40     161  HEKKGDVALP  VPYDHQRGFS  GIFEDRSYIC  KTTIGDREVD
     201  SDAYYVYRLQ  VSSINVSUNA  VQTVVRQGEN  ITLMCIVIGN
     241  EVVNFIEWTYP  RKESGRLVEP  VTDFLLDMPY  HIRSILHIPS

```

```

281 AELEDSTGYT CNVTESVNDH QDEKAINITV VESGYVRLLG
321 EVGTLQFAEL HRSRTLQVVF EAYPPPTVLW FKDNRTLGD
361 SAGEIALSTR NVSETRYVSE LTLVRVKVAE AGHYTMRAFH
401 EDAEVQLSFQ LQINVPVRVL ELSHSPDSG EQTVRCRGRG
5 441 MPQPNIIWSA CRDLKRCPRE LPPTLLGNSS EEESQLETNV
481 TYWEEEQEFE VVSTLRQLHV DRPLSVRCTL RNAVGQDTQE
521 VIVVPHSLPF KVVVISAILA LVVLTIIISLI ILIMLWQKKP
561 RYEIRWKVIE SVSSDGHEYI YVDPMLQPYD STWELPRDQL
601 VLGRTLGSAG FGQVVEATAH GLSHSQATMK VAVKMLKSTA
10 641 RSSEKQALMS ELKIMSHLGP HLNVVNLLGA CTKGGPIYII
681 TEYCRYGDLV DYLRNKHTF LQHHSDKRRP PSAELYSNAL
721 PVGLPLPSHV SLTGESDGGY MDMSKDESVD YVPMLDMKGD
761 VKYADIESSN YMAPYDNYVP SAPERTCRAT LINEPVLSTY
801 MDLVGFYSYQV ANGMEFLASK NCVHRDLAAR NVLICEGKLV
15 841 KICDFGLARD IMRDSNYISK GSTFLPLKWM APESIFNSLY
881 TTLSDVWSFG ILLWEIFTLG GTPYPELPMN EQFYNAIKRG
921 YRMAQPAHAS DEIYEIMQKC WEEKFEIRPP FSQVLVLLER
961 LLGEGYKKKY QQVDEEFLRS DHPAILRSQA RLPGFHGLRS
1001 PLDTSSVLYT AVQPNEGDND YIIPLPDPKP EVADEGPLG
20 1041 SPSLASSTLN EVNTSSTISC DSPLEPQDEP EPEPQLELQV
1081 EPEPELEQLP DSGCPAPRAE AEDSFL

```

A nucleic acid that encodes such a human PDGF beta receptor can be found in the NCBI database at accession number NM 002609, gi:15451788. See website at www.ncbi.nih.nlm.gov.

Other members of the PDGF family that may have utility when expressed by the endothelial precursor cells of the invention include vascular endothelial cell growth factor ("VEGF", sometimes also referred to as "vascular permeability factor, or "VPF") and placental growth factor ("PLGF"). Tischer et al., *Biochem. Biophys. Res. Comm.*, 165(3), 1198-1206 (1989) and Maglione et al., *Proc. Natl. Acad. Sci. USA*, 88, 9267-9271 (1991), respectively. Both VEGF and PLGF form disulfide-bonded dimers from the eight highly conserved cysteine residues that appear in the PDGF homologous region of each monomeric unit of these PDGF family members. Tischer et al. and Maglione et al., *ibid.* The receptors for VEGF and PLGF are also in the same receptor subfamily as the PDGF receptors. Consequently, these "newer" members of the PDGF family are thought to be potentially useful as therapeutic products in

wound repair and, according to the invention can be used herein to treat and prevent vascular conditions.

Hence, the endothelial precursor and other cells of the invention can be modified to express a therapeutic agent such as those described herein. Such genetic modifications can be performed by procedures available to one of skill in the art. For example, a nucleic acid encoding the therapeutic agent can be placed within an expression cassette or expression vector, and the cassette or vector can be introduced into the cell. The expression cassette can be placed within a vector to generate an expression vector.

Any vector that can replicate in a selected cell can be utilized in the invention. In general, the vector is an expression vector that provides the nucleic acid segments needed for expression of the therapeutic agent polypeptides. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

The therapeutic agent nucleic acid sequences may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. See generally, Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd edition (January 15, 2001) Cold Spring Harbor Laboratory Press, ISBN: 0879695765; Ausubel et al., Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, NY (1989)). Construction of suitable expression vectors containing a therapeutic agent can employ standard ligation techniques that are known to the skilled artisan.

The expression cassette or vector of the invention includes a promoter. A promoter is a nucleotide sequence that controls expression of an operably linked

nucleic acid sequence by providing a recognition site for RNA polymerase, and possibly other factors, required for proper transcription. A promoter includes a minimal promoter, consisting only of all basal elements needed for transcription initiation, such as a TATA-box and/or other sequences that serve to specify the site of transcription initiation. Any promoter able to direct transcription of an RNA encoding the selected therapeutic agent may be used. Accordingly, many promoters may be included within the expression cassette or vector of the invention. Some useful promoters include, constitutive promoters, inducible promoters, regulated promoters, cell specific promoters, viral promoters, and synthetic promoters. A promoter may be obtained from a variety of different sources. For example, a promoter may be derived entirely from a native gene, be composed of different elements derived from different promoters found in nature, or be composed of nucleic acid sequences that are entirely synthetic. A promoter may be derived from many different types of organisms and tailored for use within a given cell, for example, an endothelial precursor cell.

Many mammalian promoters are known in the art that may be used in conjunction with the expression cassette of the invention. Mammalian promoters often have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter may also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation (Sambrook et al., "Expression of Cloned Genes in Mammalian Cells", in: Molecular Cloning: A Laboratory Manual, 2nd ed., 1989).

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes often provide useful promoter sequences. Examples include the SV40 early promoter, mouse

mammary tumor virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallothionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated.

- 5 A mammalian promoter may also be associated with an enhancer. The presence of an enhancer will usually increase transcription from an associated promoter. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are
- 10 active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter. (Maniatis et al., Science, 236:1237 (1987); Alberts et al., Molecular Biology of the Cell, 2nd ed., 1989)). Enhancer elements derived from viruses are often times useful, because they usually have
- 15 a broad host range. Examples include the SV40 early gene enhancer (Dijkema et al., EMBO J., 4:761 (1985) and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus (Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777 (1982b)) and from human cytomegalovirus (Boshart et al., Cell, 41: 521 (1985)). Additionally, some enhancers are regulatable and
- 20 become active only in the presence of an inducer, such as a hormone or metal ion (Sassone-Corsi and Borelli, Trends Genet., 2:215 (1986); Maniatis et al., Science, 236:1237 (1987)).

- It is understood that many promoters and associated regulatory elements may be used within the expression cassette of the invention to transcribe an
- 25 encoded polypeptide. The promoters described above are provided merely as examples and are not to be considered as a complete list of promoters that are included within the scope of the invention.

- The expression cassettes and vectors of the invention may contain a nucleic acid sequence for increasing the translation efficiency of an mRNA
- 30 encoding a therapeutic agent of the invention. Such increased translation serves

to increase production of the therapeutic agent. Because eucaryotic mRNA does not contain a Shine-Dalgarno sequence, the selection of the translational start codon is usually determined by its proximity to the cap at the 5' end of an mRNA. However, the nucleotides immediately surrounding the start codon in eucaryotic mRNA influence the efficiency of translation. Accordingly, one skilled in the art can determine what nucleic acid sequences will increase translation of a polypeptide encoded by the expression cassettes and vectors of the invention.

Termination sequences can also be included in the cassettes and vectors of the invention. Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation (Birnstiel et al., Cell, 41:349 (1985); Proudfoot and Whitelaw, "Termination and 3' end processing of eukaryotic RNA", in: Transcription and Splicing (eds. B. D. Hames and D. M. Glover) 1988; Proudfoot, Trends Biochem. Sci., 14:105 (1989)). These sequences direct the transcription of an mRNA that can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 (Sambrook et al., "Expression of cloned genes in cultured mammalian cells", in: Molecular Cloning: A Laboratory Manual, 1989).

As indicated above, nucleic acids encoding the therapeutic agents can be inserted into any convenient vector. Vectors that may be used include, but are not limited to, those that can be replicated in prokaryotes and eukaryotes. For example, vectors may be used that are replicated in bacteria, yeast, insect cells, and mammalian cells. Examples of vectors include plasmids, phagemids, bacteriophages, viruses, cosmids, and F-factors. However, specific vectors may be used for specific cells types. Additionally, shuttle vectors may be used for cloning and replication in more than one cell type. Such shuttle vectors are

known in the art. The nucleic acid constructs or libraries may be carried extrachromosomally within a host cell or may be integrated into a host cell chromosome. Numerous examples of vectors are known in the art and are commercially available. (Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd edition (January 15, 2001) Cold Spring Harbor Laboratory Press, ISBN: 0879695765; New England Biolab, Beverly, MA; Stratagene, La Jolla, CA; Promega, Madison, WI; ATCC, Rockville, MD; CLONTECH, Palo Alto, CA; Invitrogen, Carlsbad, CA; Origene, Rockville, MD; Sigma, St. Louis, MO; Pharmacia, Peapack, NJ; USB, Cleveland, OH).

5 Laboratory Manual, 3rd edition (January 15, 2001) Cold Spring Harbor Laboratory Press, ISBN: 0879695765; New England Biolab, Beverly, MA; Stratagene, La Jolla, CA; Promega, Madison, WI; ATCC, Rockville, MD; CLONTECH, Palo Alto, CA; Invitrogen, Carlsbad, CA; Origene, Rockville, MD; Sigma, St. Louis, MO; Pharmacia, Peapack, NJ; USB, Cleveland, OH).

10 These vectors also provide many promoters and other regulatory elements that those of skill in the art may include within the nucleic acid constructs of the invention through use of known recombinant techniques.

A nucleic acid construct, or an expression vector can therefore be inserted into any mammalian vector that is known in the art or that is commercially available, for example, as provided by CLONTECH (Carlsbad, CA), Promega (Madison, WI), or Invitrogen (Carlsbad, CA). Such vectors may contain additional elements such as enhancers and introns having functional splice donor and acceptor sites. Nucleic acid constructs may be maintained extrachromosomally or may integrate in the chromosomal DNA of a host cell.

15 Mammalian vectors include those derived from animal viruses, which require trans-acting factors to replicate. For example, vectors containing the replication systems of papovaviruses, such as SV40 (Gluzman, *Cell*, 23:175 (1981)) or polyomaviruses, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian vectors include

20 those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the vector may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 (Kaufman et al., *Mol. Cell. Biol.*, 9:946 (1989)) and

25 pHEBO (Shimizu et al., *Mol. Cell. Biol.*, 6:1074 (1986)).

30

The invention is directed to cells that express a heterologous protein or overexpress a native protein, and nucleic acids or expression vector encoding such a heterologous or native protein. Such cells may be used for treating and preventing vascular conditions, as described herein.

5 Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include lipid-mediated transfection, dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of -the polynucleotide(s) in liposomes, biolistics, and direct microinjection of the DNA
10 into nuclei. The choice of method depends on the cell being transformed as certain transformation methods are more efficient with one type of cell than another. (Felgner et al., Proc. Natl. Acad. Sci., 84:7413 (1987); Felgner et al., J. Biol. Chem., 269:2550 (1994); Graham and van der Eb, Virology, 52:456 (1973); Vaheri and Pagano, Virology, 27:434 (1965); Neuman et al., EMBO J.,
15 1:841 (1982); Zimmerman, Biochem. Biophys. Acta., 694:227 (1982); Sanford et al., Methods Enzymol., 217:483 (1993); Kawai and Nishizawa, Mol. Cell Biol., 4:1172 (1984); Chaney et al., Somat. Cell Mol. Genet., 12:237 (1986); Aubin et al., Methods Mol. Biol., 62:319 (1997)). In addition, many commercial kits and reagents for transfection of eukaryotic cells are available.

20 Following transformation or transfection of a nucleic acid into a cell, the cell may be selected for the presence of the nucleic acid through use of a selectable marker. A selectable marker is generally encoded on the nucleic acid being introduced into the recipient cell. However, co-transfection of selectable marker can also be used during introduction of nucleic acid into a host cell.
25 Selectable markers that can be expressed in the recipient host cell may include, but are not limited to, genes that render the recipient host cell resistant to drugs such as actinomycin C₁, actinomycin D, amphotericin, ampicillin, bleomycin, carbenicillin, chloramphenicol, geneticin, gentamycin, hygromycin B, kanamycin monosulfate, methotrexate, mitomycin C, neomycin B sulfate,
30 novobiocin sodium salt, penicillin G sodium salt, puromycin dihydrochloride,

rifampicin, streptomycin sulfate, tetracycline hydrochloride, and erythromycin. (Davies et al., Ann. Rev. Microbiol., 32: 469 (1978)). Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways. Upon transfection or transformation of a cell, the cell is placed into contact with an appropriate selection agent.

Accordingly, the invention provides methods for generating and using genetically modified endothelial precursor cells that can express useful therapeutic agents.

10 **Suicide Genes for Eliminating Grafted Endothelial Cells**

The invention is also directed to genetically-modified endothelial cell precursors that express a selectable suicide gene, such as thymidine kinase (TK), which allows negative selection of grafted cells upon completion of treatment or in the event of undesired complications. TK-expressing cells can be negatively selected by the administration of gancyclovir according to methodology known in the art. Alternatively, the endothelial cell precursors can be genetically-modified to express cytosine deaminase, which causes the cells to die in the presence of added 5-fluorocytosine. The expressed gene can be lethal as a toxin or lytic agent.

Endothelial precursor cells and other cells can be genetically modified to express such "suicide genes" by available recombinant techniques, for example, as described herein.

Methods for Inducing Neovascularization

The present invention provides novel therapeutic methods employing cell therapy to treat vascular diseases, including atherosclerosis and heart disease. The invention is further directed to a method for inducing angiogenesis or neovascularization in a mammal by administering to the mammal an effective amount of a population of endothelial precursor cells, cardiac microvascular

endothelial cells (CMECs), young bone marrow cells, stem cells, embryonic stem cell lines or hematopoietic stem cells.

Neovascularization refers to the development of new blood vessels from endothelial precursor cells by any means, such as by vasculogenesis, angiogenesis, or the formation of new blood vessels from endothelial precursor cells that link to existing blood vessels. Angiogenesis is the process by which new blood vessels grow from the endothelium of existing blood vessels in a developed animal. Angiogenesis is essential for wound healing and for reproduction. Endothelial precursor cells circulate in the blood and selectively migrate, or "home," to sites of active angiogenesis (see U.S. Patent No. 5,980,887, Isner et al., the contents of which are incorporated herein by reference in their entirety).

Endothelial precursor cells may be pre-treated or co-administered with cytokines and other factors, such as, for example, PDGF, G-CSF, GM-CSF, VEGF, SCF (c-kit ligand) and bFGF, chemokines, such as SDF-1, or interleukins, such as interleukins 1 and 8. In some embodiments, endothelial precursor cells are pre-treated with such cytokines before administration to a mammal. As demonstrated herein, PDGF isoforms can also stimulate endothelial precursor cells to generate myocytes. Therefore, in some embodiments, endothelial precursor cells are pre-treated with PDGF A, PDGF B, PDGF BB or PDGF AB before administration to a mammal. Preferably, the endothelial precursor cells are pretreated with PDGF AB.

According to the invention, a platelet-derived growth factor-mediated communication exists between endothelial cells and myocytes. The PDGF dependent communication pathway comprises a series of cellular and biochemical events. Such a pathway involves cardiac myocytes that induce endothelial cells and endothelial precursor cells to express PDGF B. The PDGF B polypeptide can combine with PDGF A to generate PDGF AB. The PDGF AB protein can then stimulate endothelial cells that express the PDGF α receptor to express VEGF as well as FLK-1 and other genes. Overall, the induction of

PDGF AB expression by endothelial cells or by endothelial precursor cells promotes angiogenic function. Some variations in this pathway exist. For example, a PDGF BB dimer can form that has activity. Hence, the PDGF BB dimer can also stimulate endothelial cells to express VEGF, FLK-1 and other genes.

5 In the heart, cardiac microvascular endothelial cells (CMECs) communicate with neighboring cardiac myocytes via PDGF. Cardiac myocytes induce CMECs to express the PDGF B isoform that combines with the constitutively expressed PDGF A isoform to form the PDGF AB heterodimer. 10 This results in the induction of a cascade of molecular events that maintain vascular integrity, including the endothelial expression of vascular endothelial growth factor (VEGF) and VEGF receptor-2 (Flk-1, VEGFR-2).

According to the invention, PDGF dependent pathways mediated by endothelial precursor cells can generate cardiac myocytes from mammalian bone marrow. Precursor endothelial cells can supply PDGF to aging vascular tissues 15 that have an impaired ability to generate new blood vessels. Young adult bone marrow-derived endothelial precursor cells can recreate a platelet-derived growth factor (PDGF)-mediated communication pathway between endothelial precursor cells and cardiac myocytes and thereby contribute to the generation of 20 cardiac myocytes. While this pathway is required for cardiac vascular development and function, the pathway is lost or disrupted in older cardiac tissues and in older bone marrow. However, administration of PDGF and/or precursor endothelial cells can rescue the cardioplastic potential of the aging bone marrow.

25 According to the invention, disruption of these angiogenic pathways may lead to angiogenic defects. As recognized by the invention, this PDGF dependent communication pathway is dysfunctional in the aging heart and in the aging vasculature of mammals. "Dysfunctional" as used herein means that one or more steps in the PDGF dependent communication pathway are not

functioning properly, for example, endothelial cells in the aging heart do not express PDGF B in the presence of cardiac myocytes.

However, the invention provides methods of restoring PDGF B, PDGF AB and/or PDGF BB functions by delivery of exogenous growth factor or by recruitment of transplanted young bone marrow endothelial precursor cells can reverse the senescent impairment in cardiac angiogenic function. Thus, as provided herein, endothelial precursor cells can also help restore and stimulate cardiac myocyte generation. Endothelial precursor cells can be used to rejuvenate aging bone marrow from a mammal suffering from heart disease or other vascular diseases.

As described herein, when bone marrow is removed from older individuals, it cannot respond to myocytes and does not express PDGF B. However, when such older bone marrow cells are cultured with endothelial precursor cells, those bone marrow cells begin to express PDGF B, and begin to generate cardiac myocytes. Use of PDGF isoforms can enhance the speed at which cardiac myocytes are generated from all types of endothelial precursor cells. Use of an individual's own cells (e.g. bone marrow) avoids problems of cell typing, cell matching and the potential for immunological rejection of mismatched cells.

The invention provides cardiac myocytes exhibiting cardioplastic potential that can be derived from endothelial precursor cells obtained from a patient having senescent cardiac angiogenic function. These cardiac myocytes are obtained through a process of culturing the endothelial precursor cell in the presence of an effective amount of PDGF, for example, PDGF AB or PDGF BB. Such endothelial precursor cells can be derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

The invention further provides a method of treating a patient having senescent cardiac angiogenic function by administering endothelial precursor cells obtained from the patient having senescent cardiac angiogenic function, wherein the endothelial precursor cells were cultured in the presence of an

effective amount of PDGF prior to administration. Such PDGF can be, for example, PDGF AB or PDGF BB. Such endothelial precursor cells can be derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

5 According to the invention, the actions of PDGF extend beyond the direct regulation of blood vessels and are critical in establishing and/or maintaining an environment that permits the generation of cardiac myocytes from bone marrow stem cells. The senescent impairment in cardiac myocyte-endothelial-PDGF-B expression pathway diminishes the systemic capacity to
10 generate myocardial cells for the aging heart and contributes to the increased pathogenesis of cardiovascular disease in older persons. Since PDGF-AB enhances the generation of cardiac myocytes of bone marrow cells of all age groups, the critical downstream pathways in the precursor cells from the senescent bone marrow are likely to be intact.

15 The present invention provides experimental results demonstrating that aging-associated alterations in endothelial cells inhibit the induction of the PDGF B-dependent cardiac communication pathway that governs cardiac angiogenic function. Restoration of this pathway by administration of an exogenous growth factor such as PDGF AB, or transplantation of endothelial
20 precursor cells specifically restored cardiac angiogenic function in the aging host, and provides methods and compositions for treatment of cardiovascular disease in older individuals. The present studies were performed in unirradiated, wild type aged mice demonstrating the potential utility of bone marrow endothelial precursor cells in reconstituting endothelial function in the intact
25 vasculature without ablating the host bone marrow.

 In other embodiments, the invention provides a method of delivering PDGF B to vascular tissues that includes administering an effective amount of endothelial precursor cells to a mammal. The precursor endothelial cells become localized in cardiac tissues, and other vascular tissues, and may release
30 PDGF B to those tissues. Such release of PDGF may be sustained but need not

be. A single administration of such cells may be sufficient. Administration provides a naturally functioning cell type that may only need to be administered once or twice to generate myocytes and stimulate vascularization.

Thus, the invention provides methods for restoring senescent cardiac
5 angiogenic function by administering bone marrow endothelial precursor cells that can, for example, be recruited from young bone marrow or from PDGF-treated older bone marrow. Transplantation of endothelial precursor populations offers a simple and natural way to deliver PDGF B and angio-competent endothelial cells to sites in need of angiogenesis. Endothelial precursor cells are
10 administered as described hereinbelow. A preferred method of administration is intravascular administration.

Administration

Endothelial precursor cells may be administered in any manner used by
15 one of skill in the art to introduce the cells into the vascular system of the host. The cells may be introduced into a specific site in the vascular system to optimize delivery to a site that is known to have a vascular condition or disease. Such local delivery may avoid stimulation of inappropriate vascularization, for example, within a tumor that may be present in the mammal. However,
20 endothelial precursor cells can find their way to diseased vascular tissues, so local administration may not be needed. Moreover, endothelial precursor cells may not play a large role in tumor development because recent studies suggest tumor angiogenesis may proceed, at least in part, through a unique and unexpected pathway. Hence, concerns about stimulating tumor growth may be
25 unfounded.

Endothelial precursor cells and/or bone marrow cells may be administered by intravascular, intravenous, intraarterial, intraperitoneal, intraventricular infusion, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, or other convenient routes.
30 The cells can be washed after collection, cultured in an appropriate medium to

insure their viability and to enhance their numbers. Prior to administration, the cells can also be cultured in the presence of growth factors such as PDGF (e.g. PDGF AB), G-CSF, GM-CSF, VEGF, SCF (c-kit ligand), bFGF, chemokines such as SDF-1, or interleukins such as interleukins 1 and 8. Before
5 administration, the cells can be washed again, for example, in buffered physiological saline.

The volume of cells that is injected and the concentration of cells in the transplanted solution depend on the site of administration, the vascular disease, and the species of the host. Preferably about one-half to about five microliters is
10 injected at a time. The number of cells injected can vary, for example, about 10^2 to about 10^{10} or about 10^4 to about 10^9 cells can be injected at one time. While a single injection may be sufficient, multiple injections may also be used for prevention or treatment of vascular diseases.

Platelet derived growth factor isoforms can be administered with or,
15 without the endothelial precursor cells or young bone marrow cells of the invention. The cells may also be designed to over-express platelet derived growth factor, as described above. PDGF polypeptides can be incorporated into pharmaceutical compositions that also contain endothelial precursor cells or young bone marrow cells and that are suitable for administration to a mammal.
20 Such compositions may also contain a pharmaceutically acceptable carrier.

As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in
25 the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. The use of such media and agents for delivering cells is well known in the art. Except
30 insofar as any conventional media or agent is incompatible with the cells or

polypeptides provided herein, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

5 A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include intravenous, intraarterial, intracoronary, parenteral, subcutaneous, subdermal, or subcutaneous. Solutions or suspensions used for such administration can include other components such as sterile diluents like water for dilution, saline solutions, polyethylene glycols, glycerin, propylene
10 glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as
15 hydrochloric acid or sodium hydroxide. The composition can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For
20 intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the
25 contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the
30 maintenance of the required particle size in the case of dispersion and by the use

of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged
5 absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions to accompany the cellular suspensions can be
10 prepared by incorporating an active compound (e.g., a PDGF B polypeptide or PDGF AB protein) in the required amount in an appropriate solvent with a selected combination of ingredients, followed by filter sterilization. Generally, dispersions are prepared by incorporating an active compound into a sterile vehicle that contains a basic dispersion medium and the required other
15 ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

It is especially advantageous to formulate the cells and/or compositions
20 in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated. Each unit can then contain a predetermined quantity of the endothelial precursor cells and other components calculated to produce the desired therapeutic effect in association with the required
25 pharmaceutical carrier.

The cellular preparations and pharmaceutical compositions can be included in a kit, e.g., in a container, pack, or dispenser together with instructions for administration.

The following examples are intended to illustrate the invention and
30 should not be interpreted to limit it in any manner.

EXAMPLE 1: Endothelial Precursor Cells Restore Angiogenesis

This Example provides data illustrating that endothelial dysregulation in the PDGF communication pathway underlies the impairment in senescent cardiac angiogenic potential and that young adult BM-derived endothelial precursor cells can reverse this defect and restore cardiac angiogenesis in the aging host.

Methods**10 Molecular Studies**

Samples of the ventricular myocardium were isolated from 3 month old (n = 3) and 18 month old C57B61/L mice (n = 3). Total RNA was isolated (RNeasy and QIAshredder kits, Qiagen Valencia CA) and analyzed by RT-PCR (Hotstar Taq PCR, Qiagen) for expression of PDGF A and PDGF B as well as β -actin. Cardiac microvascular endothelial cells (CMECs) were isolated from 3 month old and 18 month old C57B61/L mice and cardiac myocytes from fetal murine hearts, as previously described. Edelberg et al. *J Clin Invest.* 1998: 102:837-43; Aird et al. *J. Cell Biol.* 1997: 138: 1117-24; Edelberg et al. *J Clin Invest.* 1998: 101: 337-43. These CMECs were then cultured in DMEM supplemented with 5% fetal calf serum, 20 U/mL heparin, 1% BME vitamins, 5 μ g/mL insulin, 5 μ g/mL transferrin, 5 ng/mL selenium, 100 μ g/mL streptomycin, and 500 μ g/mL penicillin, 4 μ g/mL endothelial growth factor and 1% endothelial cell growth supplement (all from Sigma, St. Louis MO).

Bone marrow (BM) endothelial precursor cells were isolated from 3 and 18 month old mice as previously described. Lin et al. *J Clin Invest.* 2000;105:71-77. These BM endothelial precursor cells were then cultured in DMEM supplemented with 10% fetal calf serum, and 50 μ g/mL heparin, 100 μ g/mL streptomycin, and 500 μ g/mL penicillin (all from Sigma) and 10 ng/mL vascular endothelial cell growth factor, 5 ng/mL fibroblast growth factor-2 (R & D Systems, Minneapolis MN). The endothelial cell cultures were expanded for

two passages, confirmed by Di-Ac-LDL uptake and PECAM staining, and then plated into 12 well dishes (10^5 cell/well) (Costar, Cambridge MA).

Fetal cardiac myocytes (E15.5d) were isolated and plated in 12 mm 0.4 μ m pore transwells (10^5 cell/ transwell) and then were transferred at different
5 time points (0 to 48 hours) into 3-month and 18-month old bone marrow-derived endothelial precursor cells seeded wells as described in Edelberg et al. 2002 *Circulation* 105:608-13 and Edelberg et al. *J Clin Invest.* 1998;102:837-43. As controls, cardiac microvascular endothelial cells were also isolated from 3-month and 18-month old C57B61/L mice and were cultured alone and with fetal
10 cardiac myocytes for 48 hours as described in Edelberg et al. 2002 *Circulation* 105:608-13. At the termination of the co-culture total RNA was isolated from the endothelial cell wells and RT-PCR was performed. The following sets of oligonucleotide primers were employed:

mouse PDGF A:

15 (forward): 5'TCAAGGTGGCCAAAGTGGAG3' (SEQ ID NO:7)
(reverse): 5'CTCTCTGTGACAAGGAAGCT3' (SEQ ID NO:8)

mouse PDGF B:

(forward): 5'ATCGCCGAGTGCAAGACGCG3' (SEQ ID NO:9)
(reverse): 5'AAGCACCATTGGCCGTCCGA3' (SEQ ID NO:10)

20 mouse PDGFR α

(forward): 5'ACAGAGACTGAGCGCTGACA3' (SEQ ID NO:11)
(reverse): 5'TTCCAAGAAGGAAGGAAGCA3' (SEQ ID NO:12)

mouse VEGF-164:

(forward): 5'GGATCCATGAAC TTTCTGCTGCTGTCTTGG3'
25 (SEQ ID NO:13)
(reverse): 5'TTCTGGCTTTGTCCTGTCTTTCTTTGG3' (SEQ ID NO:14)

mouse Flk-1:

(forward): 5'CAGCTTGCTCCTTCCTCATC3' (SEQ ID NO:15)
30 (reverse): 5'TCTGGAGAGCAAACCAACCA3' (SEQ ID NO:16)

mouse von Willebrand Factor

(forward): 5'TGTCCAAGGTCTGAAGAAGA3' (SEQ ID NO:17)

(reverse): 5'CAGGACAAACACCACATCCA3' (SEQ ID NO:18)

mouse PECAM

5 (forward): 5'CAAGCGGTCGTGAATGACAC3' (SEQ ID NO:19)

(reverse): 5'CACTGCCTTGACTGTCTTAAG3' (SEQ ID NO:20)

mouse β -actin

(forward) 5'GTGGGCCGCTCTAGGCACCAA 3' (SEQ ID NO:21)

10 (reverse) 5'CTCTTTGATGTCACGCACGATTTC3' (SEQ ID NO:22)

Cellular and secreted protein samples were isolated from additional endothelial cell cultures in the presence or absence of fetal cardiac myocytes as previously described. Edelberg et al. *J Clin Invest.* 1998;102:837-43. Secreted samples (50 μ l) from endothelial cells cultured alone or in the presence of cardiac myocytes were applied to Nunc maxisorp plates (Roskilde, Denmark) for 1 hour at room temperature. The samples were then washed with PBS 3 times, followed by blocking with 5% casein in PBS. Polyclonal antibodies to PDGF A (1:500, sc-128 Santa Cruz Biotechnology, Santa Cruz CA) and B, (1:300 dilution sc-7878, Santa Cruz Biotechnology), VEGF (1:200, AF 493-NA, R&D Systems), were then employed. Cellular lysate samples (50 μ L) were assayed with antibodies directed against Flk-1 (1:500, AF 644, R&D Systems), PDGFR α (1:200, AF322, R&D Systems), and PECAM (1:500 dilution 550274, BD Pharmingen San Diego CA). After washing with PBS three times the plates were developed with peroxidase-labeled donkey polyclonal antibodies to goat, rabbit, and rat IgG (1:1000, Jackson Immunoresearch Laboratories, West Grove PA) and assayed as previously described. Edelberg et al. *J Clin Invest.* 1998;102:837-43. All studies were performed a minimum of 3 times.

Cardiac Allografts Transplant Studies

Cardiac angiogenic potential was measured by employing a cardiac allograft model, which allowed testing to restore angiogenic potential while controlling the age of the cardiac tissue being vascularized.

5 The cardiac allograft procedure involved transplanting a neonatal C57B61/L (24 hr old) murine heart into the pinnae of both syngeneic young adult (3 month old) and senescent (18 month old) murine hosts as described in Aird et al. *J. Cell Biol.* 1997: 138: 1117-24; Edelberg et al. *J Clin Invest.* 1998: 101: 337-43. The recipient mice were anesthetized with Avertin 2.5% (vol/vol)
10 IP. After cleaning the dorsum of the pinna of the mouse ear with 70% ethanol, an incision penetrating only the epidermis, 2-5 mm in length, was made with a scalpel transverse to the longitudinal axis of the ear, 3-4 mm distal to its base on the skull. A small pocket between the skin and cartilage was then dissected with delicate curved forceps. The total donor neonatal heart was excised without the
15 pericardial sac and inserted into the ear pocket. Gentle pressure with the tips of the forceps was applied to the ear to express air from the pocket and facilitate the adherence between donor and recipient tissues (n = 20 three month old, n = 17 eighteen month old).

As controls, senescent mice were transplanted with inert silicon (1 x 1 x
20 2 mm³) (n = 8) or neonatal pulmonary allografts (n = 8) in place of the neonatal cardiac tissue. In addition, sets of senescent hosts were pretreated with subcutaneous pinna injections of recombinant VEGF (R&D Systems; 100 ng/20 µL PBS) (n= 12), recombinant PDGF AB (R & D Systems; 100 ng/20 µL PBS) (n=12) or vehicle alone (n = 8) 1 day prior to receiving cardiac allograft
25 transplants. In addition, at the time of cardiac or pulmonary allograft transplantation sets of young adult mice were also treated with single subcutaneous pinna injections of antibodies to neutralize PDGF AB (10 µg in 20 µL PBS, AB-20-NA, R&D Systems; n = 8 cardiac, 8 pulmonary allografts) or non-immune control rabbit IgG (10 µg in 20 µL PBS, AB-105-C, R&D Systems;
30 n = 8 cardiac, 8 pulmonary allografts). Allograft viability was scored by pinna

and transplant integrity. In addition, pinnal electrocardiograms were recorded as previously described to further document the viability of the cardiac allografts. Edelberg et al. *J Clin Invest.* 1998; 101: 337-43.

5 **Auricular Angiogenesis Studies**

Young adult (3 month old) and senescent mice (18 month old) received mid-pinnal injections of PDGF AB (100 ng/20 μ L PBS) or PBS alone (n = 8 for each group). Two days later the blood flow through both the middle and posterior auricular arteries was surgically interrupted by severing the base of the ear, thereby rendering the posterior auricular arterial circulation dependent on collateral flow from the intact anterior auricular artery, as previously described. Baker et al. 1999 *Br. J. Plast. Surg.* 52: 133-42. The functional blood flow to the posterior vascular bed was then assessed by laser Doppler with an Advance Laser Flowmeter ALF21/21D (Advance, Tokyo) as previously described.

15 Rendell et al. 1998 *Microvasc. Res.* 55: 3-13.

Following completion of the rheology studies, the mice received intracardiac injections of lysine-fixable biotinylated-dextran (2×10^6 M.W.; 50 μ L of 10^{-5} M in PBS; Molecular Probes, Eugene OR) to stain the perfused vasculature. Samples were fixed by 4% paraformaldehyde in PBS and then incubated with streptavidin-horse radish peroxidase and then developed with DAB. Histological measurements were performed with digital microscopy to assess functional vascular density of the posterior auricular vasculature as previously described. Thurston et al. 1999 *Science* 286: 2511-14.

20

25 **Bone Marrow Transplantation**

Bone marrow transplantation was performed as previously described. Spangrude et al. 1988 *Science* 241: 58-62. Briefly, 3 and 18 month old C57B1/6 mice, as well as 3-month-old B6.129S7-*Gtrosa26* (*Rosa-26*) mice were used. Friedrich et al. 1991 *Genes Dev.* 5: 1513-23. These mice were sacrificed and tibias and femurs were removed and trimmed of muscle and extraossial tissue.

30

All the cells in the *Rosa-26* express LacZ, therefore transplantation of the *Rosa-26* bone marrow into the wild-type isogenic senescent hosts facilitated the identification of the transplanted cells by X-gal staining. The bones were cut proximally and distally, and the bone marrow flushed with 2% bovine serum albumin in PBS. The cellular pellets were washed with and resuspended in PBS. The bone marrow cells were then injected into intact, unirradiated wild-type 18-month-old host C57B1/6 mice by tail vein injection with 300 μ L of cells (3 month old C57B1/6: 10^7 cells, n = 16; 10^6 , n = 12; 10^5 , n = 6; 18 month old C57B1/6, 10^7 , n = 6; 3 month old *Rosa-26*, 10^7 , n = 6). The survival rates of all mice transplanted with exogenous bone marrow was 100%. One week after bone marrow transplantation the mice received pinna cardiac allografts as described above. Seven day later mice receiving *Rosa-26* bone marrow were sacrificed and the bone marrow and exogenous cardiac tissue with surrounding pinna tissue were sectioned and stained for β -galactosidase activity as well as von Willebrand factor as previously described. Aird et al. *J. Cell Biol.* 1997: 138: 1117-24.

Results

Induction of PDGF B is Impaired in Senescent Endothelial Cells

RT-PCR analysis revealed that PDGF A was expressed in ventricular myocardial samples from both the young adult and senescent heart. See Figure 1A. PDGF B expression, however, was detected only in young adult cardiac samples (Figure 1A) suggesting that endothelial expression of PDGF B may be down regulated in the senescent heart.

Cardiac endothelial cells were isolated from both 3 and 18 month old wild-type mice and then co-cultured in the presence of fetal cardiac myocytes by using the transwell procedure described above. Endothelial cells of both young and senescent hearts constitutively expressed PDGF A. See Figures 1A and B. PDGF α -receptor (PDGFR α) was also expressed in the endothelial cells from both the young adult and senescent hearts. See Figures 1B and 1C. However,

only the young adult CMECs expressed PDGF B in the presence of the fetal cardiac myocytes. *See* Figure 1B. A significant increase in protein levels of PDGF B was observed in CMECs from 3-month-old hearts but not from 18-month-old hearts. *See* Figures 1B and 1C.

5 In addition to the differences in PDGF B expression, the expression pattern of other pro-angiogenic genes was also altered in the CMECs from aging mice. *See* Figures 1B and C. Unlike the young adult CMECs in which VEGF was induced in the co-culture with the cardiac myocytes, the senescent heart-derived endothelial cells expressed VEGF when cultured in isolation. However,
10 VEGF mRNA levels decreased in senescent CMEC when cardiac myocytes were present. Furthermore, the expression of Flk-1 (VEGFR-2), the principal mitogenic receptor for VEGF, was significantly reduced in the senescent cells. Collectively, these results suggest that a disruption in cell-to-cell communication may be a primary defect in the aging heart. In particular, aging CMECs do not
15 appear to respond to cardiac myocytes in the same manner as young CMECs.

PDGF AB Restores Cardiac Angiogenesis in Pinnal Allograft Transplants

The potential functional significance of the dysregulation in cell-to-cell
20 communication within senescent mouse heart tissues was then examined. These studies employed a syngeneic neonatal murine cardiac allograft-pinnal transplant model. Aird et al. *J. Cell Biol.* 1997; 138: 1117-24. This model effectively recreates the organ bed specific regulation of endothelial cells recruited from host peripheral vascular beds (*id.*). In these studies, endogenous PDGF AB was
25 either neutralized by injection of anti-PDGF AB antibodies or enhanced by addition of exogenous PDGF AB.

Table 1 illustrates that neutralization of PDGF AB by injection of anti-PDGF AB into the pinnae of young mice at the time of transplantation significantly reduced the viability of cardiac allografts (3/8 viable vs. 8/8 viable
30 with control antibody, $p < 0.05$). The viability of pulmonary transplant

engraftment was unaltered by neutralization of PDGF AB (8/8 viable vs. 8/8 viable with control antibody). Similarly, injection of anti-PDGFR- α antibodies at the time of transplantation significantly reduced cardiac allograft viability. Hence, PDGF AB and PDGFR- α are needed for survival of cardiac allografts.

- 5 PDGFR- α is believed to be the major receptor that mediates the PDGF pathway, whereas PDGFR- β may have only a minor role.

Table 1: PDGF AB is Needed for Survival of Pinnal Cardiac Allograft

Pinnal allograft	Antibody Pretreatment			
	IgG	Anti-PDGF-AB	Anti-PDGFR- α	Anti-PDGFR- β
Heart viability	9/9	3/8*	3/7†	7/7
Lung viability	8/8	8/8	ND	ND

10 IgG indicates immunoglobulin G; ND, not determined.

* $P < 0.05$ vs. IgG heart and anti-PDGF-AB lung trials;

† $P < 0.05$ vs. IgG and anti-PDGFR- β heart trials.

- 15 Cardiac allograft survival was markedly impaired in the aging mice as compared to the young adult mice. *See Figure 2A.* However, wound healing was preserved in the older hosts, as demonstrated by the integrity of silicon implants. The viability of the pulmonary allografts suggested that the aging-associated changes were due to diminished senescent endothelial angiogenic function.

- 20 Various molecular mediators that were observed to be down regulated in senescent cardiac tissues were then tested to ascertain whether these molecules could restore cardiac angiogenic potential in aging mice. The subcutaneous pinnal administration of VEGF failed to improve the success of cardiac transplantation in the aging mice. *See Figure 2A.* However, injection of PDGF
25 AB into senescent implantation sites restored the viability of senescent allografts to that of the young adult hosts. *See Figure 2A.* These data suggest that an aging-associated decrease in endothelial cell PDGF B gene expression underlies

the impaired function in senescent cardiac angiogenic potential observed *in vivo*. Moreover, in these studies the PDGF B expressed in the transplanted tissue appeared to be insufficient to induce effective vascularization in the senescent hosts.

5 In order to define the mechanism mediating the restoration of senescent angiogenic function, the direct effects of PDGF AB pretreatment on the pinnal vasculature of the aged mice were tested. In particular, the development of functional blood vessels in both young adult and senescent mice was assessed using the murine cardiac allograft model, where induction of angiogenesis is
10 essential for cardiac engraftment. Laser Doppler measurements demonstrated that pretreatment with PDGF AB significantly increased blood flow in both the young as well as the older hosts. *See* Figure 2B. In addition, histological assessment confirmed that the rheologic effects of PDGF AB were mediated by increasing collateral vascular density in the pinnae of both the young and
15 senescent mice. *See* Figures 2B and 2C. These results suggest that PDGF AB restores the defects in senescent cardiac angiogenic function. Moreover, the auricular studies suggest that the PDGF AB rescue of the cardiac transplants is mediated by enhancing the vascular potential in the aging murine host.

Bone Marrow Endothelial Precursor Cells Restore Cardiac Angiogenic Function

Previous work suggested that some bone marrow-derived cells might be involved in post-natal angiogenesis. Shi et al. 1998 Blood 92: 362-67; Asahara et al. 1997 Science 275: 964-67; Kalka et al. 2000 Proc. Natl. Acad. Sci. U.S.A. 97:3422-27; Takahashi 1999 Nat. Med. 5: 434-38. Bone marrow endothelial precursor cells of young mice were tested to ascertain whether they could offer a novel means of restoring the PDGF-dependent angiogenic pathways in the aging vasculature. In particular, the capacity of young bone marrow endothelial precursor cells was tested to see whether they could reconstitute the critical cardiac myocyte-mediated PDGF regulatory pathways.

Figures 3A and 3B show that PDGF B expression was induced in young bone marrow endothelial precursor cells and in young cardiac microvascular endothelial cells when these cells were co-cultured with cardiac myocytes. However, no such induction of PDGF B expression was observed in older bone marrow or cardiac microvascular endothelial cells. *See* Figure 3A. PDGF B expression was induced in these young cells within about one hour of exposure to cardiac myocytes.

Bone marrow from young LacZ⁺ *Rosa-26* mice was then transplanted intravenously into intact, unirradiated older mice. Analysis of these mice revealed that β -galactosidase-positive cells were engrafted in the senescent (older) bone marrow. *See* Figure 3C. Figure 3C provides representative photomicrographs of X-gal stained tissue sections from 18-month-old mice that had received 10^7 bone marrow cells from 3-month-old *Rosa-26* (β -galactosidase (+)) mice one week before cardiac engraftment. The transplanted young bone marrow cells were incorporated both within and around the host cardiac myocardium (Figure 3Cb-g). The bone marrow of older mice that had received young transgenic cells also stained positively for β -galactosidase (Figure 3Ca). Cells within the allograft exhibited co-staining with both von Willebrand factor and β -galactosidase (arrows highlight the transgenic cells in Figures 3Cc through

3Ce). vWF is a marker for cardiac myocyte-endothelial PDGF communication. Edelberg et al. *J Clin Invest.* 1998;102:837-43. Cells within the periallograft pinnal tissue also co-stained with both von Willebrand factor and β -galactosidase (Figure 3Cg). Cells within the allograft also stained positively for PDGF-B (Figure 3Ch). Hence, β -galactosidase-positive bone marrow endothelial precursor cells that co-stained with von Willebrand factor were recruited into the vascularization of the cardiac allografts in the peri-and intra-allograft microvasculature. See Figure 3C(c-e, g). Bone marrow endothelial precursor cells that co-stained with β -galactosidase and PDGF-B were also recruited into the vascularization of the cardiac allografts in the intra-allograft microvasculature. See Figure 3C(h).

Remarkably, transplantation of bone marrow from 3-month-old mice into intact aging murine hosts maintained the viability and restored the functioning of the exogenous cardiac tissue. See Figure 3D. However, transplantation of bone marrow from 18-month-old mice failed to reverse the aging-associated decline in cardiac angiogenic function. See Figures 3D and 3E. The restoration of the senescent vascular function was a dose dependent response in that the more young bone marrow cells transplanted, the better the viability of the allograft. See Figure 3E. These data suggest that a subpopulation of the cells that give rise to BM endothelial precursor cells mediates the *in vivo* reconstitution of the cardiac microvascular communication.

PDGF-AB Protects the Endogenous Heart From Myocardial

Infarction

Experiments were conducted to ascertain whether PDGF-AB pretreatment could significantly reduce the extent of myocardial infarction after LAD ligation. Quantification of myocardial infarction size by Masson's trichrome stain revealed that PDGF-AB reduced the size of myocardial infarction by approximately half in the young adults (Figure 4A and 4B). Similarly, the infarction size in 24-month-old heart pre-injected with PDGF-AB

was approximately half the size of infarctions in control-injected hearts (Figure 4C and 4D). Treatment at the time of coronary ligation, however, had no effect on myocardial infarction size ($15.7 \pm 3.1\%$; $n=3$). Hence, a period of pretreatment may be needed.

5

EXAMPLE 2: PDGF-AB Stimulates Cardiac Myocyte Derivation from Aging Bone Marrow

This Example provides data illustrating that aging bone marrow cells failed to generate cardiac myocytes and fail to express PDGF-B. However,
10 addition of PDGF-AB restored the cardioplastic potential of aging bone marrow cells and stimulated formation of functional cardiac myocytes that expressed myosin heavy chain and exhibited chronotropic activity *in vivo*.

Methods

15 Cell Isolation and Culture

Bone marrow cells were isolated from 3 and 18 month-old wild-type C57B1/6 mice (Harlan Sprague-Dawley, Indianapolis, Indiana; $n=3$ each). The mice were sacrificed and the tibias and femurs removed and cut proximally and distally. The bone marrow was flushed with 2% BSA in PBS. The cellular
20 pellets were washed with PBS and plated into 12-well dishes with Iscove's Modified Dulbecco's Medium supplemented with 10% fetal calf serum, 50 μ g/mL heparin, 100 μ g/mL penicillin, 100 μ g/mL streptomycin, 5ng/mL fibroblast growth factor-2, and 10ng/mL vascular endothelial growth factor. Additional studies were performed with and without supplemented of PDGF-AB
25 (R&D Systems, 10ng/mL).

Motion Analysis

Live cells were examined and recorded in real-time under phase microscopy using a Nikon TE 200 inverted microscope equipped with an Orca
30 ER digital camera and imaging software (Simple PCI, Compix). Movies were

exported in AVI format. In addition, single frames were obtained to measure systolic and diastolic diameters (D_s and D_d , respectively), in order to calculate changes in cell volume ($\Delta V = [(D_d^3 - D_s^3) / D_d^3] * 100\%$, $n=10$)

5 **Immunostaining**

At the termination of the bone marrow cultures the cells were methanol fixed and stained with monoclonal antibodies for Troponin-T (cardiac isoform) (Clone 13-11, Neomarkers). Immune complexes were visualized using a Vectastain Elite ABC-Nova Red (Vector Laboratories).

10

Molecular Studies

Total RNA was isolated from individual wells at weekly intervals for 4 weeks (RNeasy, Qiagen) and cDNA was synthesized (Sensicript Reverse Transcriptase, Qiagen). Semi-quantitative PCR was then performed in triplicate

15 using the following primers:

β -actin:

(forward) 5'GTGGGCCGCTCTAGGCACCAA3' (SEQ ID NO:23),

(reverse) 5'CTCTTTGATGTCACGCACGATTTC3' (SEQ ID

NO:24);

20

PDGF-A:

(forward) 5'TCAAGGTGGCCAAAGTGGAG3' (SEQ ID NO:25),

(reverse) 5'CTCTCTGTGACAAGGAAGCT3' (SEQ ID NO:26);

PDGF-B:

(forward) 5'ATCGCCGAGTGCAAGACGCG3' (SEQ ID NO:27),

25

(reverse) 5'AAGCACCATTGGCCGTCCGA3' (SEQ ID NO:28);

von Willebrand Factor (vWF):

(forward): 5'TGTCCAAGGTCTGAAGAAGA3' (SEQ ID NO:29),

(reverse): 5'CAGGACAAACACCACATCCA3' (SEQ ID NO:30);

PECAM:

30

(forward): 5'CAAGCGGTCGTGAATGACAC3' (SEQ ID NO:31),

(reverse): 5'CACTGCCTTGACTGTCTTAAG3' (SEQ ID NO:32);

α MHC:

(forward): 5'GGAAGAGTGAGCGGCCATCAAGG3' (SEQ ID

NO:33),

5 (reverse): 5'CTGCTGGAGAGGTTATTCCTCG3' (SEQ ID NO:34).

Cardiac Myocyte Chronotropic Analysis

In order to assess phenotypic *in vivo* cardiac chronotropic activity, bone marrow-derived cardiac myocyte aggregates derived from 3 and 18-month-old murine bone marrow cells were transplanted into syngeneic adult hosts as previously described (n=5 each). Edelberg et al. (2002) *J Appl Physiol.* 92:581-5. Briefly, sets of mice were anesthetized with Avertin IP and prepared for aggregate engraftment by subcutaneous pinnal injections of PDGF-AB (20ng/20 μ L PBS). The following day, myocyte aggregates were physically dissociated and suspended in PBS (5 x 10⁴ cells/20 μ L). These suspensions were transferred into a subdermal pinnal pocket, which was then sealed via gentle pressure with forceps. Electrocardiographic (ECG) activity of the endogenous heart and transplanted aggregates to assess chronotropic activity was performed 5-7 days post-transplantation following anesthetization with Avertin IP. ECG data was acquired as previously described. Christini et al. *Amer J Physiol.* 2001;280:H2006-2010. Following baseline recordings, chronotropic adenergetic responsiveness was measured through local administration of isoproterenol (100ng/10 μ L PBS). Statistical significance was determined by student's t-test.

25 Results

Cardioplasmic Potential of Young Bone Marrow Cells

Bone marrow cells harvested from 3-month old mice grown under conditions supporting endothelial cells developed spontaneous chronotropic activity indicative of cardiac myocyte cultures (Figure 5A). Bone marrow cells harvested from 18-month old mice grown under similar conditions did not

exhibit such chronotropic activity. The cardioplastic potential of the young bone marrow cultures was further evidenced by immunostaining for troponin T (Figure 5B), however, older bone marrow cultures did not stain positively for troponin T. These results indicate that the molecular pathways regulating the differentiation of cardiac myocytes from the aging bone marrow are impaired.

In order to develop strategies to restore the generation of cardiac myocytes from aging bone marrow cells, older bone marrow cells were exposed to factors involved in the cardiac myocyte-endothelial communication pathway. Molecular analysis revealed that PDGF isoforms were induced at the same time as cardiac myocyte-specific α myosin heavy chain (α MHC), however, vWF was expressed after PDGF and α MHC (Figure 6A). vWF is a marker for cardiac myocyte-endothelial PDGF communication. Edelberg et al. *J Clin Invest.* 1998;102:837-43. Addition of PDGF-AB increased the kinetics of cardiac myocyte generation as evidenced by α MHC expression in half the time of the bone marrow cells cultured in the absence of unsupplemented media (Figure 6B).

The *in vivo* viability of the bone marrow-derived cardiac myocytes was confirmed by transplantation of the cells into pinna of syngeneic mice. After transplantation, electropotential signals were observed from the bone marrow-derived cardiac myocyte aggregates. Greater than 80% increase in chronotropic activity was observed (226 \pm 60 vs. 120 \pm 18 depolarizations/min, baseline, $p<0.05$) (Figure 7C).

Restoring Cardioplastic Potential of Aging Bone Marrow Cells

Unlike young bone marrow, cells derived from the aging bone marrow did not express PDGF-B, α MHC, or vWF (compare Figure 6A and Figure 7A). However, as shown Figure 7B, 18-month-old bone marrow cultured in the presence of exogenous PDGF-AB did express α MHC. Moreover, the resultant cardiac myocyte aggregates derived from these cultures demonstrated phenotypic electrocardiographic activity *in vivo* (see Figures 6C and 7C).

Greater than a 60% fold increase in chronotropic activity was observed in 18-month-old bone marrow cultured in the presence of exogenous PDGF-AB (137 \pm 10 depolarizations/min, baseline,) compared to 18-month-old bone marrow cultured in the absence of exogenous PDGF-AB (83 \pm 24 depolarizations/min, baseline, $p < 0.05$)(Figure 7C).

These results indicate: (1) PDGF mediates the generation of cardiac myocytes from young bone marrow, (2) when PDGF-B induction does not occur in aging bone marrow cells, cardiac myocyte generation is impaired, and (3) addition of exogenous PDGF can stimulate and/or restore myocyte generation from bone marrow cells.

EXAMPLE 3: Transplanted Genetically-Modified Endothelial Precursor Cells Are Incorporated and Participate in Neovascularization of Syngeneic Adult Cows

This Example describes experiments where a marker gene was inserted into the genome of primary cultures of bovine fibroblasts, the fibroblasts were fused with enucleated bovine oocytes, and fetal liver cells were isolated from the resulting embryos. After these fetal liver cells were injected intravenously into syngeneic adult cows, the marker gene was detected in the vascular endothelium of the cows.

Methods

Fibroblast isolation

General procedures were employed for isolating and growing fibroblast cells from skin and lung tissue. See, for example, U.S. Patent No. 6,011,197 (Strelchenko et al.), and in U.S. Patent No. 5,945,577 (Stice et al.), the contents of both of which are incorporated herein by reference in their entirety.

The methods employed were generally as follows. Minced tissue was incubated overnight at 10 °C. in trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, N.Y.). The following day, tissue and any

disassociated cells were incubated for one hour at 37 °C. in pre-warmed trypsin-EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, N.Y.) and subjected to three consecutive washes and further trypsin incubations of one hour. Fibroblast cells were then plated in tissue culture dishes and cultured in
5 alpha-MEM medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, Utah), penicillin (100 IU/ml) and streptomycin (50 µl/ml).

Such fibroblast cells can be isolated at virtually any time in development, ranging from approximately post embryonic disc stage through adult life of the
10 animal (for example, for bovine animals, from day 12 to 15 after fertilization to 10 to 15 years of age).

Genetic modification of nuclear transfer donor cells

Culture plates containing propagating fibroblast cells were incubated in
15 trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, N.Y.) until the cells were in a single cell suspension. The cells were spun down at 500xg and re-suspended at 5 million cells per ml with phosphate buffered saline (PBS). A reporter gene construct containing the cytomegalovirus promoter operably linked to a beta-galactosidase, neomycin phosphotransferase
20 fusion gene (beta-GEO) is added to the cells in the electroporation chamber at 50 µg/ml final concentration. After providing a standard electroporation pulse, the fibroblast cells were transferred back into the growth medium (alpha-MEM medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, Utah), penicillin (100 IU/ml) and streptomycin
25 (50 µl/ml)).

The day after electroporation, attached fibroblast cells were selected for stable integration of the reporter gene by culturing them for up to 15 days in growth medium containing G418 (400 µg/ml). The neomycin phosphotransferase portion of the beta-GEO gene confers resistance to G418,
30 and cells that do not contain and express the beta-GEO gene are killed by the

selection procedure. At the end of the selection period, colonies of stable transgenic cells were present. Each colony was propagated independently of the others. Transgenic fibroblast cells were stained with X-gal to observe expression of beta-galactosidase, and genomic integration of the expression construct was confirmed by PCR amplification of the beta-GEO gene and analysis using agarose gel electrophoresis.

Cloning by nuclear transfer, with transgenic fibroblasts as nuclear donors

The stably transfected *Neo^r* fibroblast cells described above were used as nuclear donors for nuclear transfer into enucleated oocytes using procedures available in the art. See U.S. Patent No. 6,147,276 (Campbell et al.); U.S. Patent Nos. 5,945,577 and 6,235,969 of Stice et al.

Oocytes were isolated from bovine ovaries and stripped of cumulus cells to prepare for nuclear transfer. Enucleation of the recipient oocyte was performed after the oocyte attained the metaphase II stage with a beveled micropipette at approximately 18 to 24 hrs post maturation (hpm). Such enucleation can be carried out before or after nuclear transfer. Enucleation was confirmed in TL-HEPES medium plus Hoechst 33342 (3 μ g/ml; Sigma). Individual donor cells (fibroblasts) were then placed in the perivitelline space of the recipient oocyte, and the oocyte and donor cell were fused together to form a single cell (a nuclear transfer unit) using a single one fusion pulse consisting of 120 V for 15 μ sec to the nuclear transfer unit in a 500 μ m gap chamber. In some experiments, nuclear transfer and electrofusion was performed at 24 hrs post maturation. The nuclear transfer units were then incubated in CR1aa medium.

Nuclear transfer units were activated as described in co-pending U.S. Application No. 09/467,076 (Cibelli et al.), filed December 20, 1999, the contents of which are incorporated herein by reference in their entirety. Following activation, the nuclear transfer units were washed and cultured under conditions that promote growth of the nuclear transfer unit to from 2 to about 400 cells. In particular, the nuclear transfer units were transferred to the wells of

plates containing a confluent feeder layer of mouse embryonic fibroblasts as described in U.S. Patent No. 5,945,577. After multicellular embryos were formed, they were implanted into cows to develop into fetal animals.

In the case of human applications, this example would apply in every
5 respect with the exception that the human endothelial precursor cell would be produced from cloned human embryonic stem cells derived from pre-implantation (<14 day-old) embryos. The nuclear transfer units can be incubated until they reach the blastocyst stage, and the inner cell mass (ICM) cells of these nuclear transfer units can be isolated and cultured in the presence or absence of a
10 feeder layer to generate pluripotent or totipotent embryonic stem cells.

The fetal calves were aborted, and fetal liver cells were isolated and injected intravenously into syngeneic adult cows. That is, in each transplant, the cloned, transplanted cells were administered to the same animal from which the donor fibroblasts used to generate the transplanted cells were originally obtained.
15 At 414 days post transplantation, arterial tissue was removed from one of the treated cows (animal # 31) and endothelial cells from the arterial tissue were isolated and expanded. The endothelial cell outgrowths were analyzed to detect cells containing the transgene (*Neo^r*).

20 **Transplanted cells participate in neovascularization in a transplant recipient**

Bovine fibroblasts were isolated and stably transfected with a recombinant DNA construct comprising a *Neo^r* gene under control of a CMV promoter; stably transfected fibroblasts were cloned by nuclear transfer to
25 generate multicellular bovine embryos; and these were implanted into cows to develop into fetal animals, as described above. Transgenic fetuses were aborted and fetal liver/bone marrow cells were isolated and intravenously injected into an adult cow (animal # 33), also as described above.

Matrigel (BD) was defrosted overnight in 4°C, and aliquots of 20 ml
30 were mixed with 2 micrograms heparin (Sigma) and 4 micrograms human

vascular endothelial growth factor (PeproTech). Matrigel is a basement membrane extract. It is polymerizable into a rigid stable gel upon heating at 24°-37°C. A more complete discussion of Matrigel can be found in U.S. Pat. No. 4,829,000. The Matrigel mixture was injected with pre-cooled syringe
5 subcutaneously at a suitable site. During injection of the Matrigel, the needle was kept in place for approximately 5 min. while lifting up the skin with the needle point, in order to allow the Matrigel to solidify as a plug.

After 14-21 days the animal was sacrificed and the Matrigel plugs are removed and cut into two portions. One part of the plug was fixed in 4%
10 paraformaldehyde, embedded in paraffin, sectioned, and H & E stained. Sections were examined by light microscopy, and the number of blood vessels that have formed in the plug was evaluated. The other part of the Matrigel plug was digested by addition of Dispase (Invitrogen) for 5-10 minutes at 37°C until the gel was liquefied and cells were released. The cells were expanded in-vitro
15 and were evaluated to determine their cell type and to detect cells that have the *Neo^r* transgene. Other tissues of the cow, e.g., bone marrow, endothelium, lymph node, etc. were also analyzed to detect and identify cells that have a *Neo^r* transgene.

20 Results

Transplanted cells are incorporated into the vascular endothelium of a transplant recipient

At 414 days post transplantation, arterial tissue was removed from one of the cows treated with *Neo^r* endothelial precursor cells (animal # 31) and
25 endothelial cells from the arterial tissue were isolated and expanded. The endothelial cell outgrowths were analyzed to detect cells containing the transgene (*Neo^r*). Of five separate endothelial cell outgrowths, one of them (20%) was positive for the *Neo^r* gene.

Bone marrow stem cells of a cow that received the transplant were
30 isolated and cultured to form primary hematopoietic colonies. Eight pools were

made of cells from the primary hematopoietic colonies, each pool consisting of cells from about 40 colonies, and the pools were tested for the presence of cells containing the *Neo^r* transgene. Two of the eight pools tested positive for the *Neo^r* transgene, indicating that approximately 1-2% of the hematopoietic stem cells in the cow's bone marrow were derived from the transplanted transgenic cells. *Neo^r* positive cells were also detected in the lymph nodes of the cow that received the transplant.

These results indicate that transplanted transgenic, nuclear transfer-derived hematopoietic stem cells are not rejected by a syngeneic recipient mammal that has an intact and functioning immune system, even though they have heterologous mitochondria. These results also demonstrate that the transplanted cells become established in the bone marrow and lymph tissue of the transplant recipient and give rise to differentiated endothelial precursor cells that incorporate into the vascular endothelium of the transplant recipient. Thus, immune rejection of stem cells need not be a problem, and stem cells can repopulate the vascular endothelium to facilitate repair and rejuvenation of the aging vascular system of mammalian tissues.

EXAMPLE 4: Transplantation and engrafting of genetically modified murine endothelial cells

This Example shows that fetal liver hematopoietic stem cells possess the ability to transdifferentiate and repair damaged tissue (infarcted myocardium) at the site of injection while expressing a transgenic marker gene, and differentiate into vascular endothelium.

Methods

The methods employed generally involve isolating somatic cells from 129/SV EV mice and genetically modifying the cells by insertion of an expression construct directing expression of the LacZ gene into their genomic DNA. Such transgenic murine cells were then used as nuclear donor cells, and

cloned, transgenic fetal mice carrying the LacZ gene were produced by somatic cell nuclear transfer.

The methods employed are described in more detail below. Methods for cloning mice by somatic cell nuclear transfer are provided in Wakayama et al., 1998, "Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei," *Nature* 394:369-374, the contents of which are incorporated herein by reference in their entirety. Methods employed for culturing murine blastocysts produced nuclear transfer to generate an isogenic embryonic stem cell line, for genetically modifying the nuclear transfer-derived embryonic stem cells by homologous recombination, and for inducing the genetically modified embryonic stem cells to differentiate in vitro to form hematopoietic precursors that can be therapeutically engrafted into mice in need of the transplant were similar to those described in Rideout, 3rd, et al., "Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy," 2002, *Cell*, 109(1):17-27; the contents of which are incorporated herein by reference in their entirety.

Nuclear transfer and embryo culture

Cloned 129/Sv-ROSA26::LacZ fetuses were produced by piezo-actuated microinjection (Prime Tech, Japan) essentially as described previously (Wakayama et al., 1998, *nature* 394:369-74; Wakayama and Yanagimachi, 1999, *Nature Genetics*, 22:127). Nucleus donor cells were isolated from primary cultures derived from tail tip biopsies of 8-week-old 129/Sv-ROSA26::lacZ males and cultured at 37°C in 5% (v/v) CO₂ in humidified air in gelatin-coated 3.5 cm² flasks for 10-14 days in Dulbecco's modified ES medium (DMEM; GIBCO) supplemented with 15% (v/v) FCS. Immediately prior to use, cells were dissociated by treatment with trypsin and the reaction quenched by the addition of DMEM prior to washing three times in PBS. A 1 - 3 ml aliquot of the resultant nucleus donor cell suspension was mixed with a 10 - 20 ml drop of HEPES-buffered CZB containing polyvinylpyrrolidone (Mr 360,000) and the

nuclei were injected into enucleated B6D2F1 oocytes within 1 h of mixing. After approximately one hour, nuclear transfer oocytes were activated by exposure to SrCl_2 for 1h and then incubated in KSOM (Specialty Media, NJ) lacking SrCl_2 at 37°C in 5% (v/v) CO_2 in humidified air (Wakayama et al., 1998). Cleaved (2-cell) embryos were transferred the next day (E1.5) to the oviducts of pseudopregnant CD1 surrogate mothers. Cloned fetuses recovered at 11 to 13 days gestation were used as a source of liver cells.

Isolation of c-Kit positive liver cells

On two separate occasions cloned embryos were obtained. In the first instance, a group of four embryos at 12-13 days gestation were obtained and in the second instance, two embryos at 11 and 13 days gestation were obtained. Embryonic liver cells were obtained by mechanically disaggregating embryos through a 40 micrometer cell strainer (Becton Dickinson, Franklin Lakes, NJ) and selected for c-kit+ cells. A total of 1.67×10^7 nucleated cells were obtained after disaggregation from the first group, and 5.8×10^6 cells were obtained from the second group. Cells were incubated with PE-conjugated anti-c-kit antibody (BD Pharmingen, San Diego, CA), and sorted on a MoFlow cell sorter (Dako Cytomation, Fort Collins, CO). In the first study 5×10^5 c-kit+ cells were obtained, and in the second study, 1.95×10^5 c-kit+ cells were obtained. The cells were suspended in 1ml phosphate buffered saline with 10% fetal calf serum at 4°C.

The c-kit-positive fetal liver hematopoietic stem cells were injected into adult mice suffering from myocardial infarction. Prior to injection of the fetal cells, myocardial infarction was induced in adult 129 SV EV mice by occlusion of the left descending coronary artery near its origin. Four to six hours later, approximately 10,000 c-kit-positive fetal liver cells were injected at each of two sites in opposite regions of the border zone, adjacent to the non-contracting dead portion of the left ventricular wall (n=10). Control groups consisted of untreated infarcted mice (n=10) and sham-operated animals (n=9).

Results

The three groups of treated, untreated and sham-operated mice were sacrificed one month after surgery or sham operation. Infarct size was measured by the fraction of myocytes lost by the entire left ventricle inclusive of the interventricular septum. The dimension of the infarct was similar in the two groups of mice exposed to permanent coronary artery ligation. In the treated animals, infarct size was $56 \pm 5\%$, for which the total number of myocytes was $2.72 \pm 0.30 \times 10^6$, and the number of myocytes lost was $1.54 \pm 0.13 \times 10^6$. In the untreated animals, infarct size was $54 \pm 6\%$, for which the total number of myocytes was $2.72 \pm 0.30 \times 10^6$, and the number of myocytes lost was $1.48 \pm 0.15 \times 10^6$.

In the untreated mice at one month after surgery, the healing process was completed and the area of infarcted myocardium was a compact scarred area. Analysis of the connective tissue present in the scarred area identified the presence of both collagen type III and collagen type I.

In contrast, myocardial regeneration within the infarct occurred in all mice injected with fetal liver cells. Newly formed myocytes were recognized by the expression of β -sarcomeric actin, cardiac myosin heavy chain, connexin 43, and N-cadherin antibody labeling. Importantly, the developed myocardium also possessed coronary capillaries, which were identified by factor VIII antibody and Griffonia simplicifolia lectin labeling. Coronary resistance arterioles were numerous and were detected by α -smooth muscle actin antibody staining. The arterioles and capillaries contained in their lumen red blood cells, which were stained by TER-119 antibody. The presence of red blood cells in the lumen strongly suggested that the generated vessels were connected with the primary coronary circulation. Labeling with β -galactosidase antibody documented that these new structures, including myocytes, endothelial cells and smooth muscle cells, were all β -galactosidase-positive and were of fetal liver cell origin.

LacZ gene expression was assayed at the site of injection in both the myocardium and the endothelium. Most of the LacZ gene-containing cells that were detected in the repaired tissue were myocardial, but endothelial cells containing the LacZ were detected as well.

5 Quantitatively, in mice treated with fetal liver cells, the band of regenerated myocardium had an average volume of $7.4 \pm 3.0 \text{ mm}^3$ and occupied $38 \pm 11\%$ of the infarcted scarred tissue. Together, $8.2 \pm 2.6 \times 10^6$ new myocytes were formed. The volume of these myocytes varied from 200 to 2,700 μm^3 , averaging $690 \pm 160 \mu\text{m}^3$. There were 250 ± 60 capillaries and 30 ± 10 arterioles per mm^2 of reconstituted myocardium. The extent of tissue replacement reduced the size of the infarct by 18%, from 56 to 46% of the entire left ventricle. The reduction of infarct size was not sufficient to attenuate the remodeling of the post-infarcted heart. Chamber diameter, chamber volume, the wall thickness-to-chamber radius ratio and the left ventricular mass-to-chamber volume ratio were not statistically different from those evaluated in infarcted untreated mice. However, measurements of hemodynamic parameters obtained before sacrifice in the closed-chest preparation showed an improvement of left ventricular end-diastolic pressure in infarcted mice with myocardial regeneration induced by the injection of fetal liver cells. Additionally, diastolic wall stress was reduced by nearly 30% in this group. Thus, fetal liver cells regenerate infarcted myocardium and ameliorate the diastolic properties of the infarcted ventricle.

References

- 25 1. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest.* 1999;103:697-705.
2. Malouf NN, Coleman WB, Grisham JW, et al. Adult-derived stem cells from the liver become myocytes in the heart in vivo. *Am J Pathol.* 2001;158:1929-35.

3. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701-5.
4. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. 2001;107:1395-402.
5. Hakuno D, Fukuda K, Makino S, et al. Bone Marrow-Derived Regenerated Cardiomyocytes (CMG Cells) Express Functional Adrenergic and Muscarinic Receptors. *Circulation*. 2002;105:380-386.
6. Rafii S, Shapiro F, Pettengell R, et al. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood*. 1995;86:3353-63.
7. Davis TA, Lee KP. Ex vivo expansion of primitive murine hematopoietic progenitor cells on porcine endothelial cells. *Transplant Proc*. 1997;29:2005.
8. Mohle R, Salemi P, Moore MA, Rafii S. Expression of interleukin-5 by human bone marrow microvascular endothelial cells: implications for the regulation of eosinophilopoiesis in vivo. *Br J Haematol*. 1997;99:732-8.
9. Rafii S, Mohle R, Shapiro F, et al. Regulation of hematopoiesis by microvascular endothelium. *Leuk Lymphoma*. 1997;27:375-86.
10. Yourey PA, Gohari S, Su JL, Alderson RF. Vascular endothelial cell growth factors promote the in vitro development of rat photoreceptor cells. *J Neurosci*. 2000;20:6781-8.
11. Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol*. 2000;425:479-94.
12. Wang T, FitzGerald TJ, Haregewoin A. Differential expression of nitric oxide synthases in EGF-responsive mouse neural precursor cells. *Cell Tissue Res*. 1999;296:489-97.

13. Edelberg JM, Tang L, Hattori K, et al. Young Adult Bone Marrow-Derived Endothelial Precursor Cells Restore Aging-Impaired Cardiac Angiogenic Function. *Circ. Res.* 2002;90:e89-e93.
14. Edelberg JM, Aird WC, Wu W, et al. PDGF mediates cardiac
5 microvascular communication. *J Clin Invest.* 1998;102:837-43.
15. Edelberg JM, Lee SH, Kaur M, et al. Platelet-Derived Growth Factor-AB Limits the Extent of Myocardial Infarction in a Rat Model: Feasibility of Restoring Impaired Angiogenic Capacity in the Aging Heart. *Circulation.* 2002;105:608-613.
- 10 16. Weinsaft JW, Edelberg JM. Aging-Associated Changes in Vascular Activity – A Potential Link to Geriatric Cardiovascular Disease. *Amer J Geriatric Cardiology.* 2001;10:348-354.
17. Edelberg JM, Jacobson JT, Gidseg DS, et al. Enhanced myocyte-based biosensing of the blood-borne signals regulating chronotropy. *J Appl*
15 *Physiol.* 2002;92:581-5.
18. Christini DJ, Walden J, Edelberg JM. Direct biologically-based biosensing of dynamic physiological Function. *Amer J Physiol.* 2001;280:H2006-2010.
19. Betsholtz C. Role of platelet-derived growth factors in mouse
20 development. *Int J Dev Biol.* 1995;39:817-25.
20. Ataliotis P, Mercola M. Distribution and functions of platelet-derived growth factors and their receptors during embryogenesis. *Int Rev Cytol.* 1997;172:95-127.

25 All publications and patents are incorporated by reference herein, as though individually incorporated by reference. The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

WHAT IS CLAIMED:

1. A method for treating a vascular condition in a mammal comprising administering to the mammal a therapeutically effective amount of endothelial precursor cells.
2. The method of claim 1, wherein the endothelial precursor cells express platelet derived growth factor or bind platelet derived growth factor.
3. The method of claim 1, wherein expression of platelet derived growth factor is induced in the endothelial precursor cells when the endothelial precursor cells are co-cultured with cardiac myocytes.
4. The method of claim 1, wherein the endothelial precursor cells express c-kit.
5. The method of claim 1, wherein the vascular condition is a myocardial infarction.
6. The method of claim 5, wherein a myocardial infarction's size is reduced after administration of the endothelial precursor cells.
7. The method of claim 1, wherein the vascular condition is atherosclerosis.
8. The method of claim 1, wherein the vascular condition is ischemia, tachycardia, congestive heart failure, peripheral vasculature disorder, hypertension, stroke, thrombosis, arrhythmia or tachycardia.
9. The method of claim 1, wherein the mammal is a human.
10. The method of claim 1, wherein the administration is intravascular,

intravenous, intraarterial, intraperitoneal, intraventricular infusion, via infusion catheter, via balloon catheter, via bolus injection, or via direct application to tissue surfaces during surgery.

11. The method of claim 1, wherein the therapeutically effective amount of endothelial precursor cells is about 10^2 to about 10^{10} endothelial precursor cells.

12. The method of claim 1, wherein the therapeutically effective amount of endothelial precursor cells is about 10^4 to about 10^9 endothelial precursor cells.

13. The method of claim 1, wherein the endothelial precursor cells are exposed to platelet derived growth factor AB prior to administration to the mammal.

14. The method of claim 1, wherein the endothelial precursor cells are syngeneic endothelial precursor cells originally obtained from the mammal to be treated.

15. The method of claim 14, wherein the mammal is pre-treated with G-CSF, GM-CSF, VEGF, SCF, bFGF, SDF-1, interleukin 1 or interleukin 8 before the endothelial precursor cells were obtained.

16. The method of claim 1, wherein the endothelial precursor cells are derived from bone marrow, peripheral blood, umbilical cord blood, liver tissue or fat.

17. The method of claim 1, wherein the endothelial precursor cells are derived from an embryonic stem cell line.

18. The method of claim 1, wherein the endothelial precursor cells are derived from at least one nuclear transfer unit formed in vitro by fusion of an enucleated oocyte with a somatic cell from the mammal.
19. The method of claim 1, wherein the endothelial precursor cells are derived from an inner cell mass of a blastocyst generated in vitro.
20. The method of claim 1, wherein the endothelial precursor cells comprise a heterologous DNA encoding a therapeutic agent that can be expressed in the endothelial precursor cells.
21. The method of claim 20, wherein the therapeutic agent is a platelet derived growth factor polypeptide.
22. The method of claim 21, wherein the platelet derived growth factor polypeptide comprises SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6.
23. The method of claim 20, wherein the therapeutic agent is a platelet derived growth factor receptor.
24. The method of claim 23, wherein the platelet derived growth factor receptor comprises SEQ ID NO:35 or SEQ ID NO:36.
25. The method of claim 20, wherein the therapeutic agent is a cytokine, a growth factor, a hormone, streptokinase, tissue plasminogen activator, plasmin, urokinase, an anti-thrombotic agent, an anti-inflammatory agent, a metalloproteinase inhibitor or a nematode-extracted anticoagulant protein.

26. A method for treating or preventing a myocardial infarction in a patient having or at risk for developing a myocardial infarction, said method comprising administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

27. The method of claim 26, wherein the agent that restores a PDGF B dependent communication pathway is at least one of PDGF AB, PDGF BB, PDGF A, PDGF B, stem cells, young bone marrow cells, endothelial precursor cells, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or a small molecule.

28. The method of claim 26, wherein administration is by intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.

29. The method of claim 27, wherein the stem cells, young bone marrow cells or endothelial precursor cells are genetically modified to express a heterologous protein or to over-express a native protein.

30. The method of claim 29, wherein the heterologous protein or the native protein is a platelet derived growth factor polypeptide.

31. The method of claim 30, wherein the platelet derived growth factor polypeptide comprises SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6.

32. The method of claim 29, wherein the heterologous protein or the native protein is a platelet derived growth factor receptor.

33. The method of claim 32, wherein the platelet derived growth factor receptor comprises SEQ ID NO:35 or SEQ ID NO:36.
34. The method of claim 29, wherein the heterologous protein or the native protein is a cytokine, a growth factor, a hormone, streptokinase, tissue plasminogen activator, plasmin, urokinase, an anti-thrombotic agent, an anti-inflammatory agent, a metalloproteinase inhibitor or a nematode-extracted anticoagulant protein.
35. A method for reducing the size of a myocardial infarction in a patient at risk for developing a myocardial infarction, said method comprising administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.
36. The method of claim 35, wherein the agent that restores the PDGF B dependent communication pathway is at least one of PDGF AB, PDGF BB, PDGF A, PDGF B, stem cells, young bone marrow cells, endothelial precursor cells, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or a small molecule.
37. The method of claim 35, wherein the size of the myocardial infarction is measured by the extent of myocardial necrosis.
38. The method of claim 35, wherein the route of administration is by intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.
39. The method of claim 36, wherein the stem cells, young bone marrow cells or endothelial precursor cells are genetically modified to express a

heterologous protein or over-express a native protein.

40. A method of restoring cardiac angiogenic function in an patient having senescent cardiac angiogenic function, said method comprising administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

41. The method of claim 40, wherein the agent that restores a PDGF B dependent communication pathway is at least one of PDGF AB, PDGF BB, PDGF A, PDGF B, stem cells, young bone marrow cells, endothelial precursor cells, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or a small molecule.

42. The method of claim 40, wherein the administration is by intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.

43. The method of claim 41 wherein the stem cells, young bone marrow cells or endothelial precursor cells are genetically modified to express a heterologous protein or to over-express a native protein.

44. A method of restoring vascular function in an patient having peripheral vasculature disorder (PVD) said method comprising administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

45. The method of claim 44 wherein the agent that restores a PDGF B dependent communication pathway is at least one of PDGF AB, PDGF BB, PDGF A, PDGF B, stem cells, young bone marrow cells, endothelial precursor

cells, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or a small molecule.

46. The method of claim 44, wherein the administration is by intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.

47. The method of claim 45, wherein the stem cells, young bone marrow cells or endothelial precursor cells are genetically modified to express a heterologous protein or over-express a native protein.

48. A method of restoring vascular function in or near the brain of a patient in need of such restoration, said method comprising administering to the patient a therapeutically effective amount of an agent that restores a PDGF B dependent communication pathway.

49. The method of claim 48, wherein the agent that restores a PDGF B dependent communication pathway is at least one of PDGF AB, PDGF BB, PDGF A, PDGF B, stem cells, young bone marrow cells, endothelial precursor cells, epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, or a small molecule.

50. The method of claim 48, wherein administration is by intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.

51. The method of claim 49, wherein the stem cells, young bone marrow cells or endothelial precursor cells are genetically modified to express a

heterologous protein or over-express a native protein.

52. The method of claim 48, wherein the patient is suffering or has suffered a stroke.

53. A method of treating cardiovascular dysfunction, said method comprising administering to a patient suffering from said dysfunction, a therapeutically effective amount of cardiac myocytes, wherein said cardiac myocytes are derived from autologous stem cells and wherein said stem cells have been cultured in the presence of PDGF B.

54. The method of claim 53, wherein the stem cells are derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

55. The method of claim 53, wherein the cardiovascular dysfunction is at least one of myocardial infarction, ischemia, peripheral vasculature disorder (PVD), stroke, arrhythmia, tachycardia, or heart failure.

56. The method of claim 53, wherein the stem cells are genetically engineered to express a heterologous protein or overexpress a native protein.

57. The method of claim 53, wherein the cardiac myocytes are genetically engineered to express a heterologous protein or overexpress a native protein.

58. The method of any of claims 53-57, wherein the administration is by intravascular, intravenous, intraarterial, intraperitoneal or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.

59. A method of restoring cardiac angiogenic function in a patient having

senescent cardiac angiogenic function, said method comprising administering to said patient a therapeutically effective amount of cardiac myocytes, wherein said cardiac myocytes are derived from autologous stem cells and wherein said stem cells have been cultured in the presence of PDGF AB.

60. The method of claim 59, wherein the stem cells are derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

61. The method of claim 59, wherein the cardiovascular dysfunction is at least one of myocardial infarction, ischemia, peripheral vasculature disorder (PVD), stroke, arrhythmia, tachycardia, or heart failure.

62. The method of claim 59, wherein the stem cells are genetically engineered to express a heterologous protein or overexpress a native protein.

63. The method of claim 59, wherein the cardiac myocytes are genetically engineered to express a heterologous protein or overexpress a native protein.

64. The method of any of claims 59-63, wherein the administration is by intravascular, intravenous, intraarterial, intraperitoneal, or intraventricular infusion, stent, infusion catheter, balloon catheter, bolus injection, direct application to tissue surfaces during surgery, oral or topical administration.

65. A method of restoring cardioplastic potential of bone marrow cells obtained from a patient having senescent cardiac angiogenic function, said method comprising administering to a culture of said bone marrow cells an effective amount of PDGF AB.

66. A method of restoring cardioplastic potential of stem cells obtained from a patient having senescent cardiac angiogenic function, said method comprising

administering to a culture of said stem cells, an effective amount of PDGF AB.

67. A method of increasing the kinetics of cardiac myocyte derivation from bone marrow cells obtained from a patient having senescent cardiac angiogenic function, said method comprising administering to a culture of said bone marrow cells an effective amount of PDGF AB.

68. A method of increasing the kinetics of cardiac myocyte derivation from stem cells obtained from a patient having senescent cardiac angiogenic function, said method comprising administering to a culture of said stem cells an effective amount of PDGF AB.

69. The method of any one of claims 65-68 wherein the stem cells are derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

70. Cardiac myocytes exhibiting cardioplastic potential and derived from stem cells obtained from a patient having senescent cardiac angiogenic function, said cardiac myocytes obtained through a process of culturing said stem cells in the presence of an effective amount of PDGF AB.

71. The cardiac myocytes of claim 70 wherein the stem cells are derived from bone marrow, peripheral blood, umbilical cord blood, organs, tissue, or fat.

1/10

Fig.1A

Cardiac Ventricular Myocardium

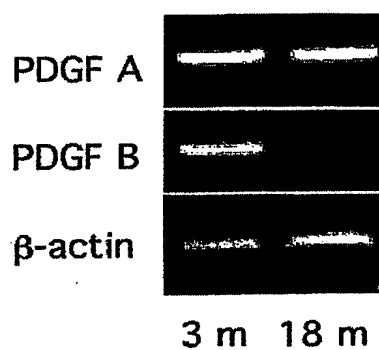


Fig.1B

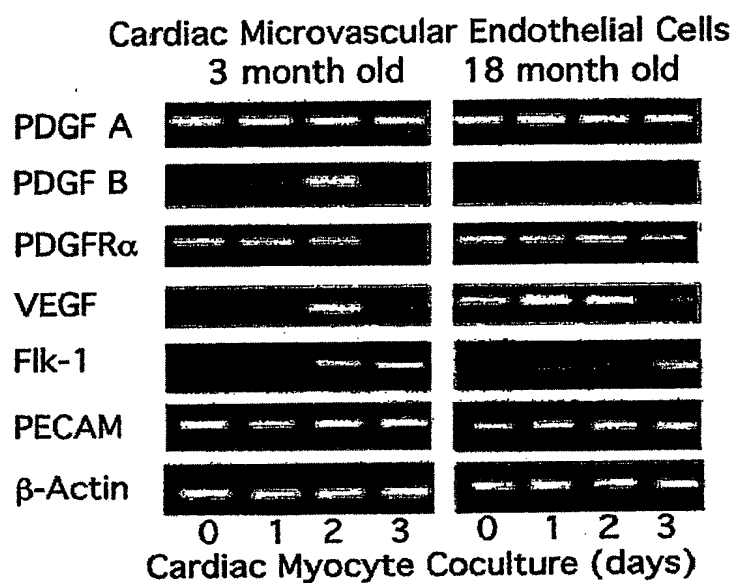
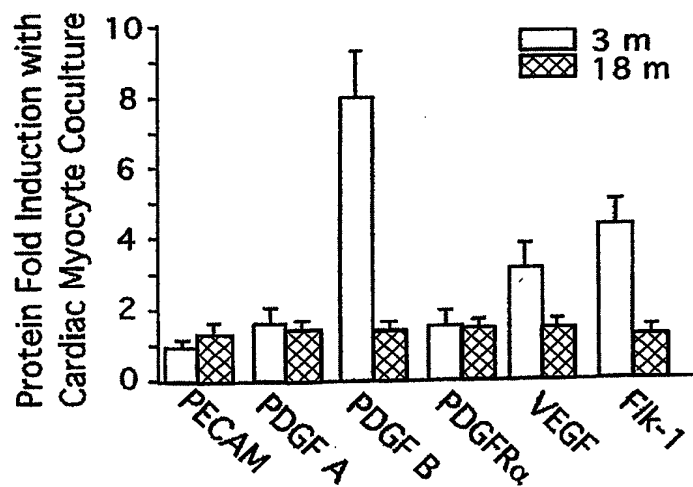


Fig.1C



2/10









	18 month old				3 month old			
Transplant								
Pretreatment	heart	heart	lung	silicon	heart	heart	heart	heart
Viability	PDGF AB 100%**	VEGF 17%	- 75%	- 100%	- 12% *	- 95%	-	-

Fig.2A

3/10

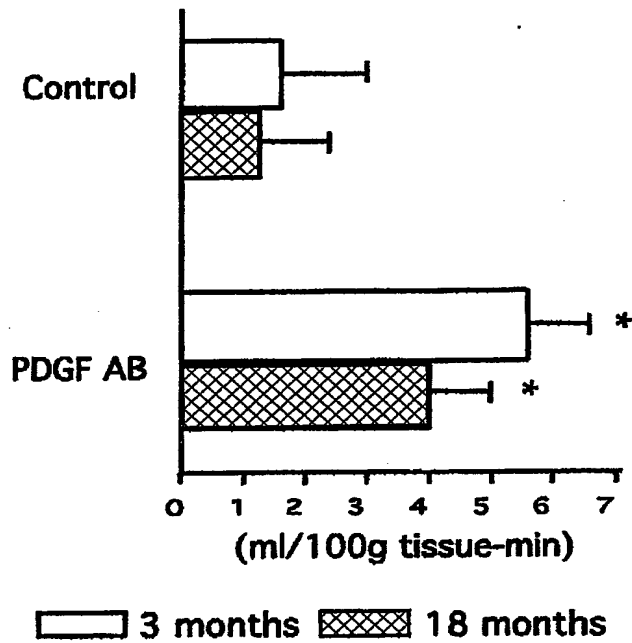
Pinna Blood Flow

Fig.2B

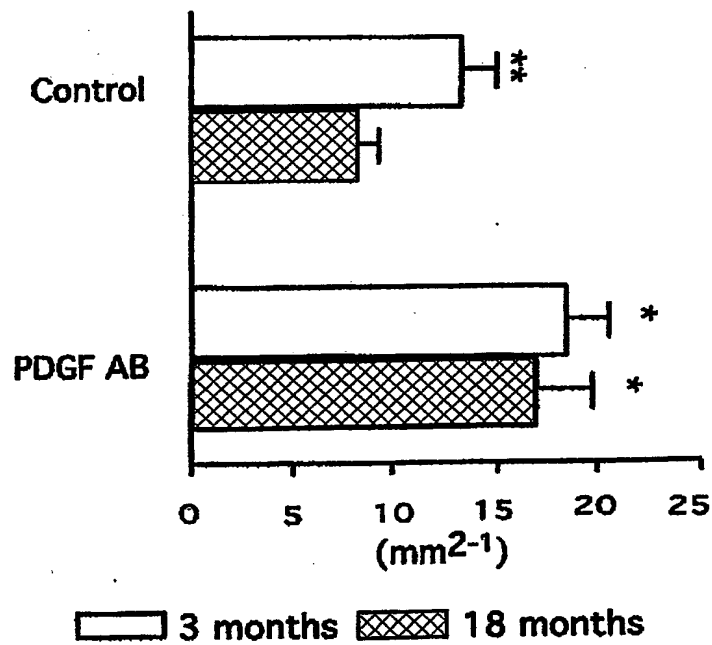
Vascular Density

Fig.2C

4/10

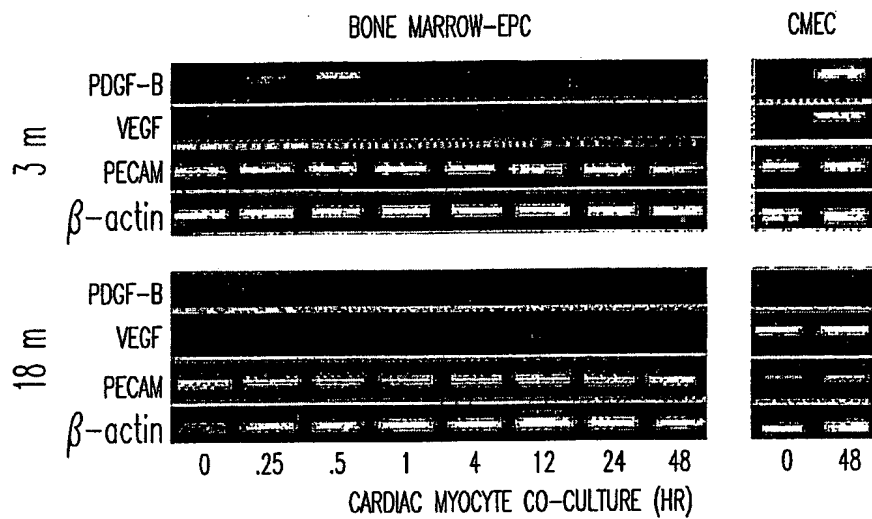


Fig.3A

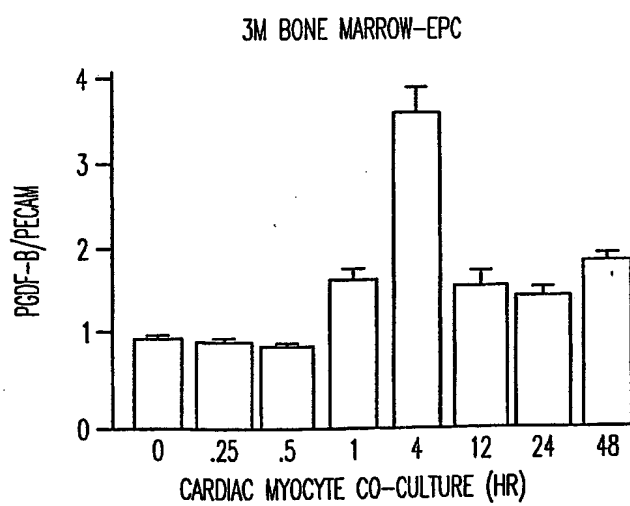


Fig.3B

5/10



Fig.3C

6/10

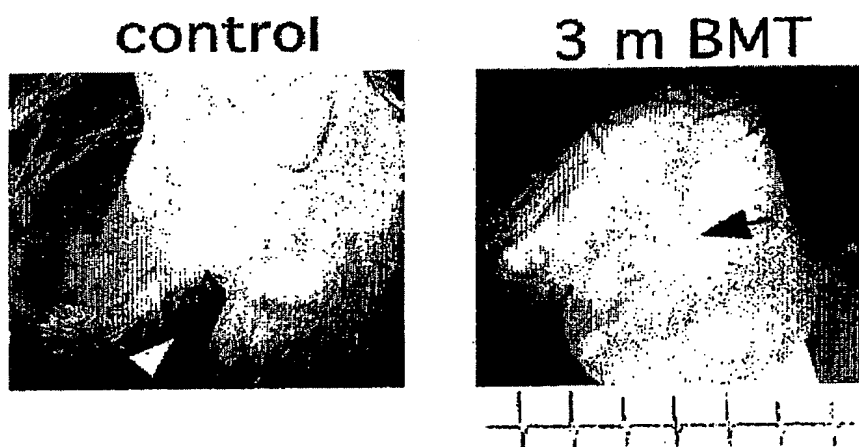


Fig.3D

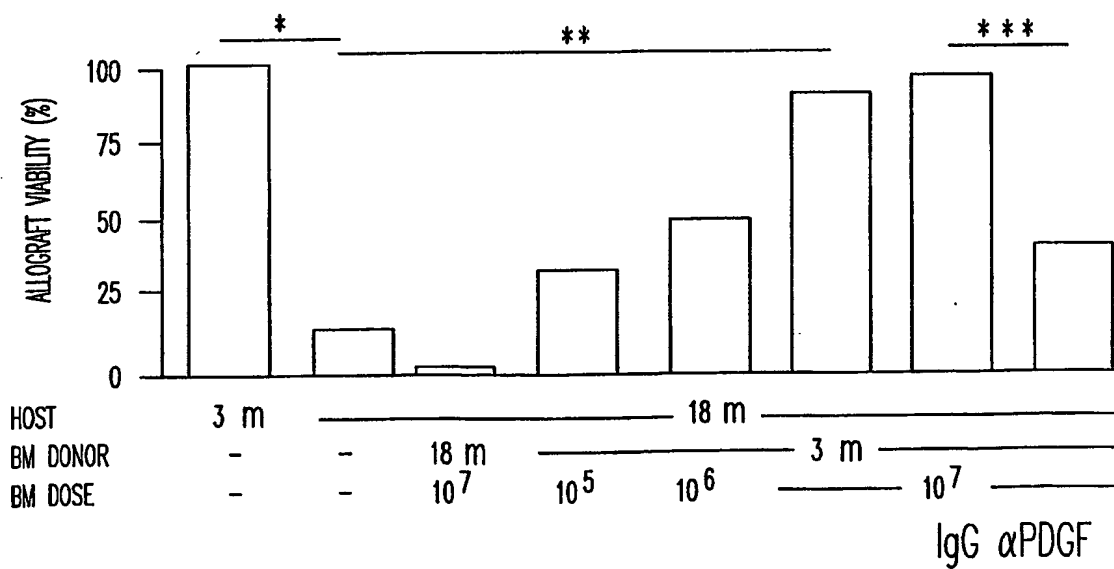
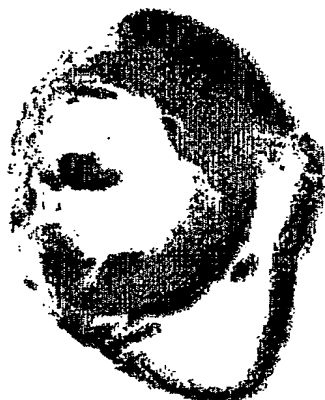


Fig.3E

7/10



PDGF



CONTROL

Fig.4C

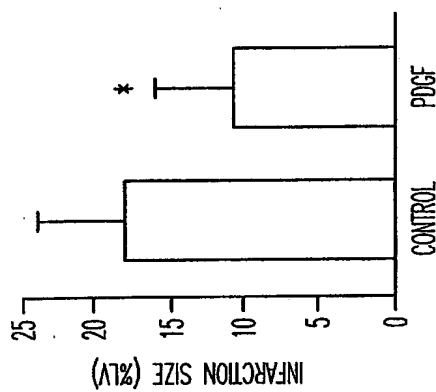


Fig.4D



PDGF



CONTROL

Fig.4A

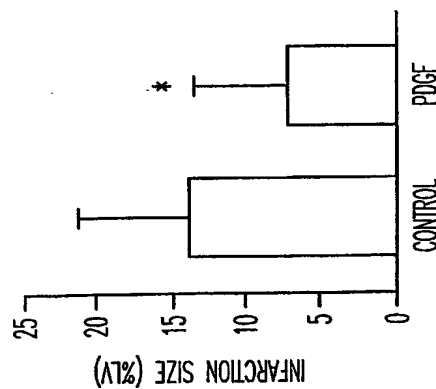


Fig.4B

8/10



Fig.5

9/10

Fig.6A

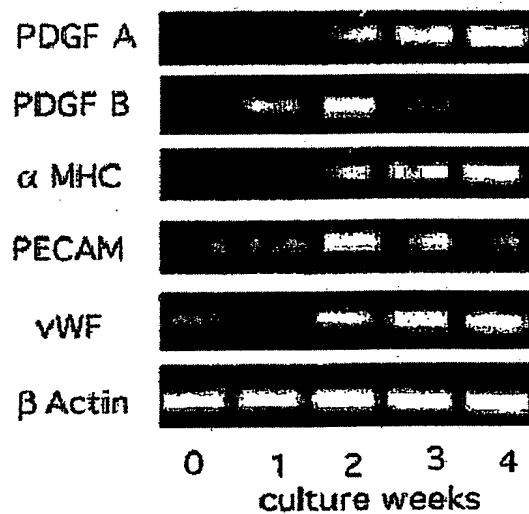


Fig.6B

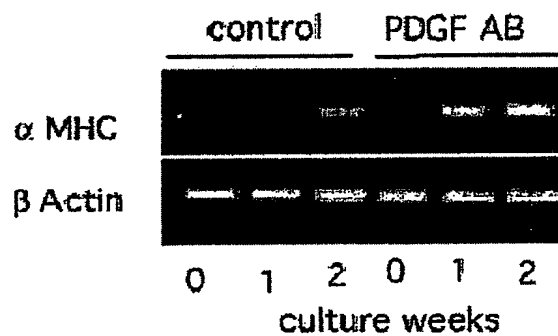
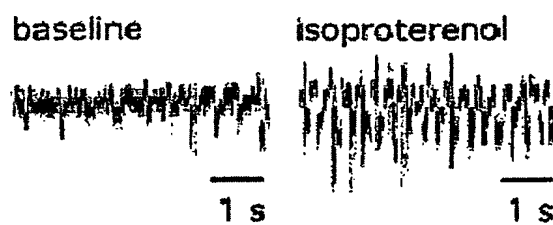


Fig.6C



10/10

Fig.7A

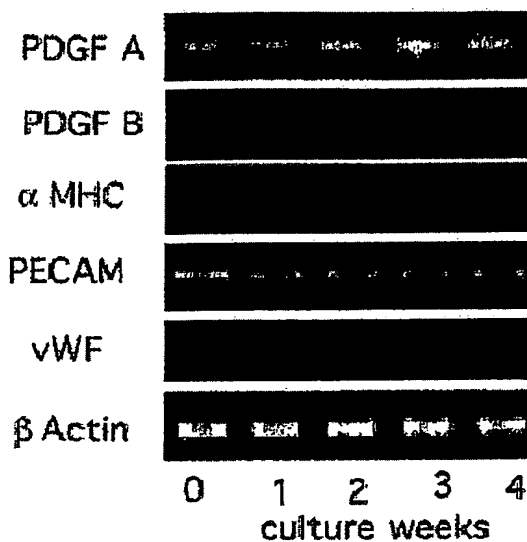


Fig.7B

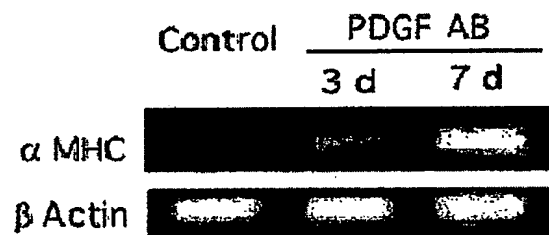
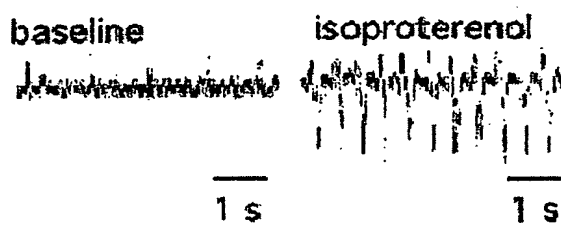


Fig.7C



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☒ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.